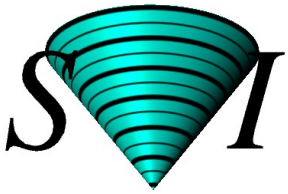
Huygens Essential User Guide

Scientific Volume Imaging



Huygens Essential User Guide

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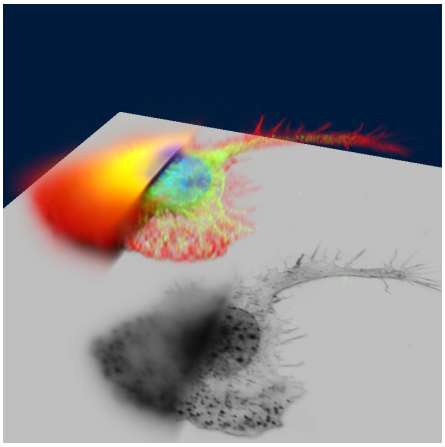
Cover illustration:
Macrophage recorded by Dr. James Evans (White-head Institute, MIT, Boston MA, USA) using widefield microscopy, as deconvolved with Huygens.



At the right the same dataset again: macrophage fluorescently stained for tubulin (yellow/green), actin (red) and the nucleus (DAPI, blue). Left part: original data; right part: as deconvolved with the classical Maximum Likelihood Estimation method (MLE).

The image was visualized using the Simulated Fluorescence Process (SFP) volume rendering package from Scientific Volume Imaging.

Image in figures 21, 23, 26 and 27: isolated Rat Hepatocyte couplet recorded by Dr. Permsin Marbet at the Department of Anatomy of the University of Basel, Switzerland (head: Prof. Lukas Landmann), as deconvolved with Huygens.

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1. What is Huygens Essential?

Huygens Essential is an image processing software package tailored for deconvolution of microscopic images. Its wizard driven user interface guides you through the process of deconvolving microscopic images. Huygens Essential is able to deconvolve a wide variety of images ranging from 2D widefield (WF) images to 4D multi-channel multi-photon confocal images. To facilitate comparison of raw and deconvolved data, or results from different deconvolution runs the Essential is equipped with a dual 4D slicer tool. You can also render 3D images and animations with its powerful visualization tools.

Based on the same image processing engine (the compute engine) as Huygens Professional, Huygens Essential combines the quality and speed of the algorithms available in Huygens Professional with the ease of use of a wizard driven intelligent user interface.

Huygens Essential is based on cross-platform technology. It is available on various Microsoft Windows operating systems, Linux for Intel or AMD based systems, MAC OS X, IBM AIX and SGI's Irix 6.5. For AIX and Irix 64 bit multiprocessing versions are also available.

2. Installing Huygens Essential

You can download Huygens Essential from the SVI website: `http://www.svi.nl`

Mac OS X

Go to the folder were you downloaded the distribution and double-click on it. It will be extracted by "StuffIt Expander" to a `.pkg` file, which will be placed in the same directory. Double click this `.pkg` file, and follow the installation wizard.

Microsoft Windows

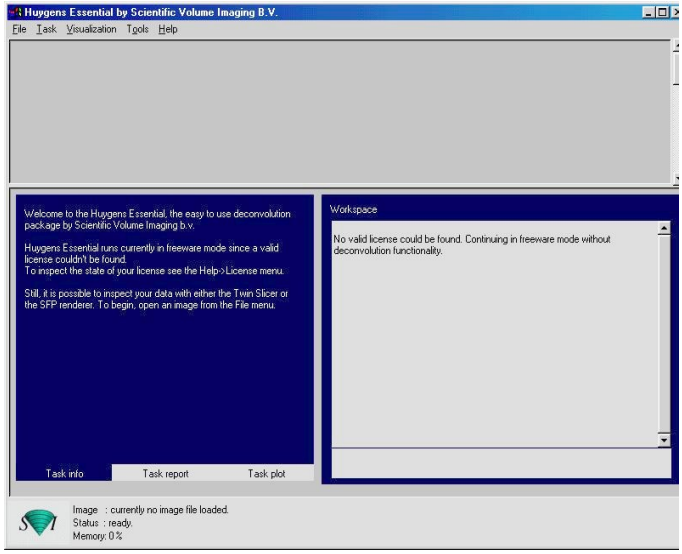

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
Welcome to the Huygens Essential, the easy to use deconvolution package by Scientific Volume Imaging b.v.
Huygens Essential runs currently in freeware mode since a valid license couldn't be found.
To inspect the state of your license see the Help->License menu.
Still, it is possible to inspect your data with either the Twin Slicer or the SFP renderer. To begin, open an image from the File menu.
Workspace
No valid license could be found. Continuing in freeware mode without deconvolution functionality.
Task info
Task report
Task plot
Image : currently no image file loaded.
Status : ready.
Memory: 0 %

Figure 1. ***The start-up window on Microsoft Windows.*** *If no license string is installed the software runs in 'Freeware mode'. You can find your computer's ID number by using* HELP > ABOUT

You have received an executable file for installation, for instance a file named `Huygense280.exe`. Place this file on your desktop and double click its icon to start the installation. During installation the directory `C:\Program files\SVI\` will be created by default. After completion the Huygens Essential and Huygens Scripting icons appear on your desktop. Double clicking on the Huygens Essential icon starts the program; the start-up window will be displayed (Figure 1).

Linux

The Huygens Essential Linux distribution is a 'rpm' file, for instance `huygens-2.7.0-p7.rpm`. Open a Unix shell, go to the directory were this file is located, become superuser and type: `rpm -ivh --force huygens-2.7.0-p7.rpm`

After installing the software type `essential` in a shell to start the software. A directory `/usr/local/svi` will be created; initialization scripts will be installed in `/usr/local/bin`.

SGI Irix 6.5

Currently the Irix distribution is a single 'tardist' file containing various components. By default all components are installed. Become superuser and type:

```
swmgr -f dist65-2.7.0-p7.tardist
```

Press START in the Software Manager window.

After installing the software type `essential` in a shell to start the software. The program will display the start-up window (Figure 1). A directory `/usr/local/svi` will be created; the executables will be installed in `/usr/local/bin`.

IBM AIX 5.2

Log in as root on your workstation or ask your system administrator to do so. Huygens for AIX is distributed in a tar file. Go to the directory where you downloaded it to. After unpacking the distribution file with `tar xvf my_aixfile.tar` you will find three new files: `svi.tar.Z`, `README.AIX` and a shell script `AIX-install.sh`. To install the software you have to run the file `AIX-install.sh` by typing `./AIX-install.sh`. This script will unpack the `svi.tar.Z` file under `/usr/local` and then will do some post installation tasks and verification. After a successful installation it will print the message "OK." to the screen.

A directory `/usr/local/svi` will be created; the executables will be installed in `/usr/local/bin`.

After the installation

After a first-time installation there is not yet a license available. Still, you can start the software. Without a license it will run in 'Freeware mode'. Among others, this gives you access to the License tools in the HELP menu. The next section explain how to obtain and install a license string.

On AIX, Irix and Linux start the software by clicking its icon or by typing `essential` into a Unix shell. On Mac OS X and Windows click its icon. The software will open in Freeware mode and display the start-up window (Figure 1).

The license string

The license key used by all SVI software is a single string per licensed package. It may look as follows:

```
HuEss-2.7-wcnp-d-tv-emnps-eom2008Dec31-e7b7c623393d708e-
{frank@svi.nl}-4fce0dbe86e8ca4344dd
```

At startup Huygens Essential searches for a license file `huygensLicense` which contains a license string. This license string is provided by SVI via e-mail. Installing the license string is the same for all platforms, though on Linux, Irix and AIX only the superuser can do this.

Obtaining a license string

If you are not upgrading from a previous installation it is likely that a license is not yet available. To enable us to generate a license string for you we need the *fingerprint* of your computer, the *system ID* number. If you have not done so already, start Huygens Essential. The system ID is displayed in the HELP > ABOUT dialog (Figure 2). Send it to `sales@svi.nl`, and you will be provided with a license string. To prevent any typing error use the COPY button to save the ID to the clipboard. You can print it into your mail message with the EDIT > PASTE menu item of your mail program.

In this dialog box you can also find a button to CHECK FOR HUYGENS UPDATES on our company server.

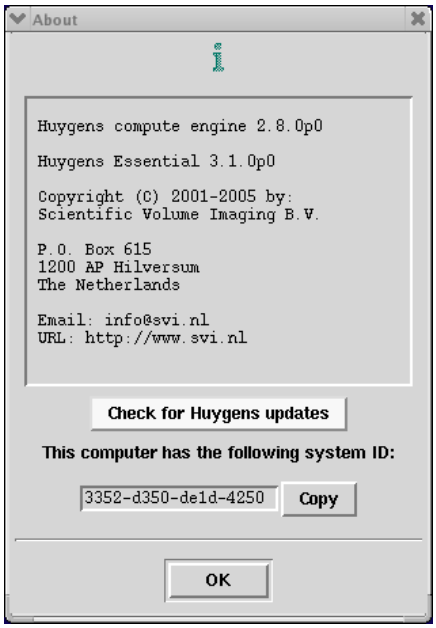


Figure 2 ***The* HELP > ABOUT *window****. The system ID is shown at the bottom.*

Installing the license string

Select the license string in your email message and copy it to the clipboard using EDIT > COPY in your mailing program. Start Huygens Essential and go to HELP > LICENSE: a dialog box pops up (Figure 3). Then press the ADD A NEW LICENSE button and a new window will pop up (Figure 4). Paste the license string into the text field[1]. Complete the procedure using the ADD LICENSE to add the string to the `huygensLicense` file. Please try to avoid typing the license string by hand: any small typing error will invalidate the license. With an invalid license, the software will remain in Freeware mode.

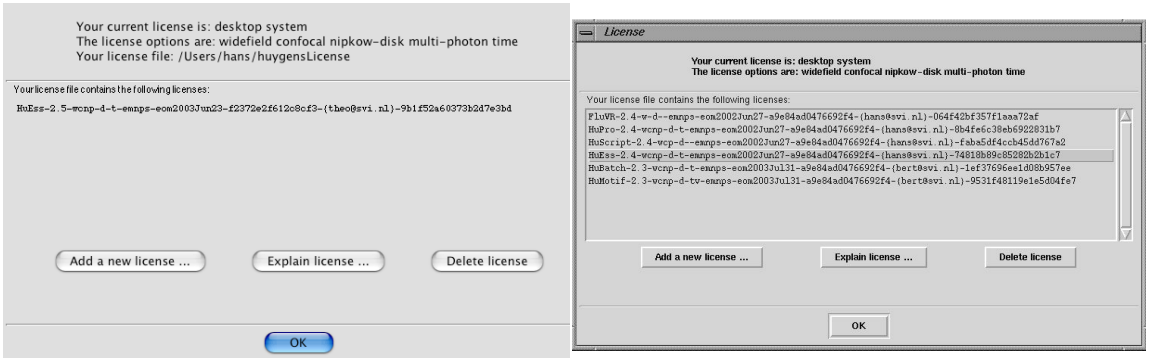


Figure 3. ***The License manager dialog.*** *Left: as displayed on Mac OS X; right: as displayed on Irix. The License manager allows you to add, delete and troubleshoot licenses.*

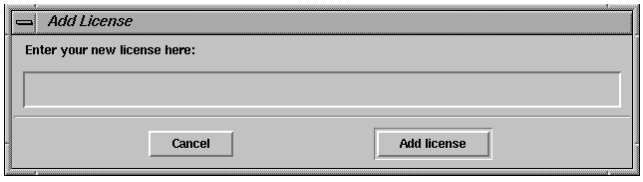


Figure 4. ***The Add License dialog box****.*

1 Use your operating system's generic copy / paste operations: *Mac OS X:* apple-c / apple-v; *Windows:* control-c / control-v; *Linux, Irix, Aix:* on most common desktops copying is done simply by marking the text area with the mouse, and pasting by either middle mouse click or control-v.

Location of the license file

The license string is added to the file `huygensLicense` in the `svi` directory. On the different supported platforms this is located in:

- AIX, Irix and Linux: `/usr/local/svi`
- Mac OS X: depends on where you installed the software. A typical example is `/Applications/SVI`
- Windows: `C:\Program Files\SVI\`

On AIX, Irix and Linux and Mac OS X an alternative location is the user's home directory. On OS X this is especially convenient when updating frequently.

Restart Huygens Essential to activate the new license. This will enable the deconvolution or PSF distiller functionality.

Trouble shooting license strings

The license string as used by SVI has the same appearance on all supported platforms. For each product[2] you need to have a license string installed. Select a license string in the License manager and press the EXPLAIN LICENSE button. All details for the current license will be listed. If you run into licensing problems you may use this information to analyze the problem. See *License string details* on page 41.

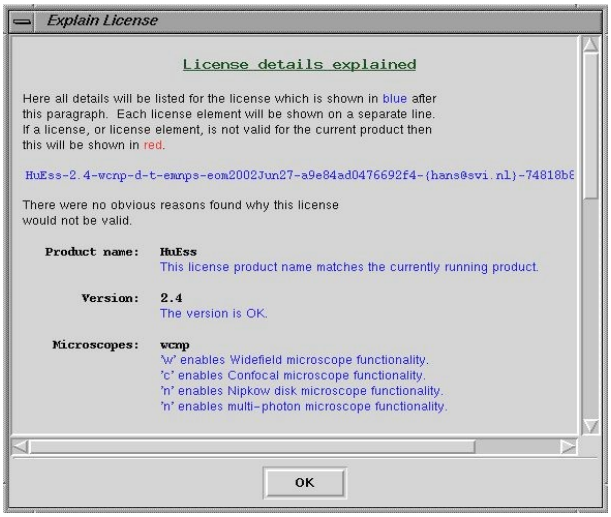

Explain License
License details explained
Here all details will be listed for the license which is shown in blue after this paragraph. Each license element will be shown on a separate line. If a license, or license element, is not valid for the current product then this will be shown in red.
HuEss-2.4-wcnp-d-t-emnps-eom2002Jun27-a9e84ad0476692f4-(hans@svi.nl)-74818b8
There were no obvious reasons found why this license would not be valid.
Product name: HuEss
This license product name matches the currently running product.
Version: 2.4
The version is OK.
Microscopes: wcnp
'w' enables Widefield microscope functionality.
'c' enables Confocal microscope functionality.
'n' enables Nipkow disk microscope functionality.
'n' enables multi-photon microscope functionality.
OK

Figure 5. ***The License details.***

Updating the software

Download the new version from the SVI website at `http://www.svi.nl`. Proceed with the installation as explained above.

Do not uninstall the old version as this will delete your license string. On Mac OS X make sure you make a backup of the license string in a safe place before you remove the previous installation.

Removing the software

- Irix: Open the Software Manager, select MANAGE INSTALLED SOFTWARE, and mark the packages you wish to remove.
- Linux: To remove Huygens Essential type as root: `rpm -e huygens`
- Mac OS X: Drag the installation to the waste basket.
- Microsoft Windows: Clicking START in your Windows desktop and select: PROGRAMS > HUYGENS ESSENTIAL > UNINSTALL.

Removing the software will also cause your license string to be removed. If you prefer to uninstall your current version prior to installing a newer one, be sure to store your license string in a safe place.

2 Huygens Essential, Huygens Professional and Huygens Scripting.

System requirements for Huygens Essential

Windows and Linux

Huygens Essential and Huygens Scripting run on the following Windows operation systems: Windows 2000, NT, 2003 Server and XP.

Linux: RedHat and SuSE distributions. Since Linux versions evolve rapidly best consult SVI's `http://www.svi.nl` web page to see which Linux distributions are currently supported.

A standard Ethernet card is required to provide your computer with a system ID.

- Processor: Pentium III or IV (Intel) or Athlon (AMD).
- Recommended RAM memory: 512 MB or larger (to run larger images like 512*768*50 voxels).
- Graphics card: any fairly modern card will do.

Mac OS X

Mac OS X 10.2 or higher running on a G4 processor or higher. 512 MB or more RAM.

SGI Irix, IBM AIX

Huygens Essential and Huygens Scripting run on all SGI equipment running Irix 6.5 on a MIPS R5000 processor or higher, IBM AIX 5.2 equipment with a Power4 processor or higher. The recommended RAM size is 512 MB or larger.

Running the program

Mac OS X

You can find the program icon in the installation directory. Clicking it will start Huygens Essential and open the main window (Figure 6). You can also run the program by typing `essential` at a shell prompt.

Windows

The Windows installation procedure has automatically placed an Huygens Essential icon on your desktop. Clicking it will start Huygens Essential and open the main window (Figure 6).

Linux

On Linux you can start Huygens Essential by typing `essential` at a shell prompt. It will start Huygens Essential and opens the main window (Figure 6).

If the shell is unable to find this command then typing the full path should help:

```
/usr/local/bin/essential
```

If this still does not help then Huygens Essential has not been installed correctly.

In Linux KDE desktop you may also start Huygens Essential from the Application menu and in the Gnome desktop from the Main Menu.

Irix

On Irix you can start Huygens Essential by typing `essential` at a shell prompt which will start Huygens Essential and opens the main window (Figure 6).

If the shell is unable to find this command then typing the full path should help:

```
/usr/sbin/essential
```

If this still does not help then Huygens Essential has not been installed correctly.

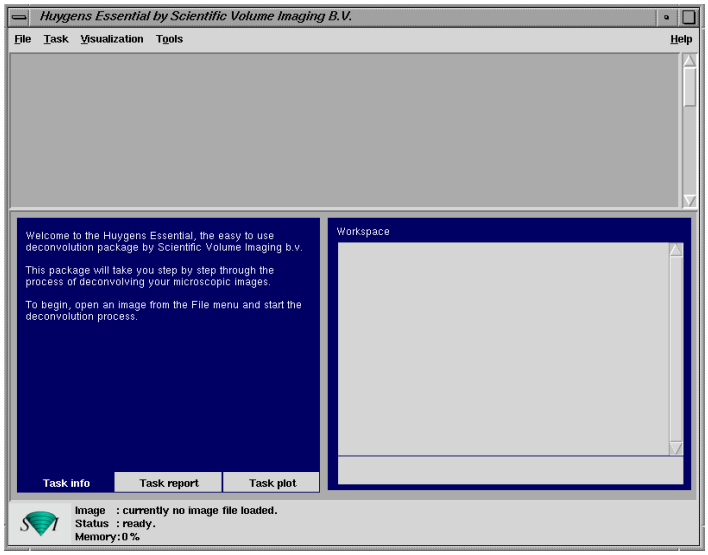

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
Welcome to the Huygens Essential, the easy to use deconvolution package by Scientific Volume Imaging b.v.
This package will take you step by step through the process of deconvolving your microscopic images.
To begin, open an image from the File menu and start the deconvolution process.
Workspace
Task info
Task report
Task plot
Image : currently no image file loaded.
Status : ready.
Memory: 0 %

Figure 6. ***The start-up window.*** *The gray area in the upper part is the work area where the thumbnail representation of the original image and its individual channels will be placed. The blue window in the left will show help text, task reports and supporting information during the various processing stages. In the gray bottom right field different dialog boxes will appear during processing. Also intermediate deconvolution results are shown here. The bottom bar is a status area.*

Adding to the shell search path

Users of the csh or tcsh shell can add the `/usr/local/bin` or `/usr/sbin/` directory to their shell search path by adding the following line to the `.cshrc` file in their home directory:

`set path=(/usr/local/bin $path)` or `set path=(/usr/sbin $path)`

You can inquire your shell by typing: `echo $shell`

Support on installation

If you find any problem in installing the program or the licenses that you could not solve with the guidelines here included, please search the knowledge database or contact SVI on the addresses on page 45.

3. The image restoration process

The processing stages

Huygens Essential guides you through the process of microscopic image deconvolution (also referred to as 'restoration') in several stages. Each stage is composed of one or more tasks. While proceeding each stage is briefly described in the bottom-left *Task Info* window pane. The stages progress is indicated at the right side of the status bar (see Figure 15 on page 13). Additional information can be found in the HELP > QUESTIONS and HELP > DICTIONARY (Figure 7).

The following steps and stages are to be followed:

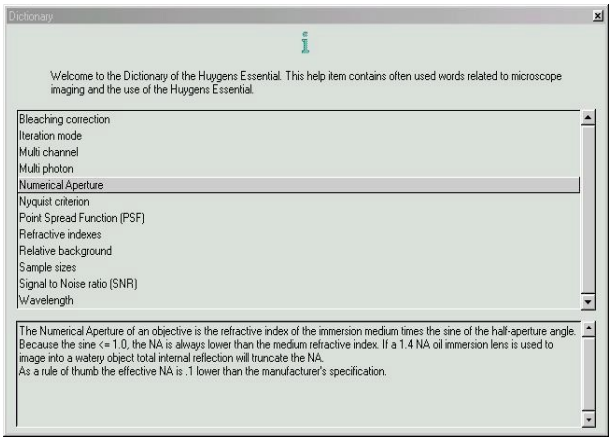

Dictionary
Welcome to the Dictionary of the Huygens Essential. This help item contains often used words related to microscope imaging and the use of the Huygens Essential.
Bleaching correction
Iteration mode
Multi channel
Multi photon
Numerical Aperture
Nyquist criterion
Point Spread Function (PSF)
Refractive indexes
Relative background
Sample sizes
Signal to Noise ratio (SNR)
Wavelength
The Numerical Aperture of an objective is the refractive index of the immersion medium times the sine of the half-aperture angle. Because the sine <= 1.0, the NA is always lower than the medium refractive index. If a 1.4 NA oil immersion lens is used to image into a watery object total internal reflection will truncate the NA.
As a rule of thumb the effective NA is .1 lower than the manufacturer's specification.

Figure 7. ***The Dictionary.*** *The* DICTIONARY *and the* QUESTIONS *from* HELP *give additional information.*

- Opening an image. A demo image (the `faba64.ics/ids` file pair) is placed in the `images` subdirectory of `svi`.
- stage P: The Preprocessing stage: loading an image, converting data sets, parameter check and cropping.
- stage 1: Tune the parameters. This stage will be skipped when you entered from the preprocessing stage. In the preprocessing stage you have already checked the parameter settings for the intelligent cropper - intelligent since it uses a-priori knowledge for setting the optimal cropping boundaries automatically. You will enter this stage from the latest one when clicking the <<RESTART button. This is useful if you wish to fine tune your parameters for the best deconvolution result, in particular when you like to set your parameters slightly different when using multi-channel images (see page 17).
- stage 2: Inspecting the image histogram.
- stage 3: Background estimation.
- stage 4: The deconvolution run.
- saving the result.

The different stages will be explained below

Loading an image

Select OPEN from the FILE menu to enter the file browser and move to the directory where your images are stored. Select the image to be deconvolved, e.g. the `faba64.ics/ids` file pair in the `images` subdirectory of `svi`.

Several formats from microscope vendors are supported. If you have TIFF images to be processed please read *TIFF file series naming convention* on page 43 for the naming convention in order to be able to read a multi-dimensional image as a whole.

When the file is read successfully you can either press START DECONVOLUTION to begin processing your image or you can convert your data set with the TOOLS button.

If you have loaded a bead image you also can proceed selecting START PSF DISTILLER and proceed with generating a Point Spread Function (PSF, see page 14) from measured beads (See *The PSF Distiller* on page 27).

A special license is needed in order to launch the PSF Distiller.

You can OPEN ADDITIONAL images for reference purposes, but only the one named 'original' will be deconvolved during the guided restoration.

Preprocessing

Converting a data set (optional)

Before you press the START DECONVOLUTION button you can convert a 3D stack into time series images (Convert XYZ to XYZT) or vice versa, or you can convert a 3D stack into a time series of 2D images (XYZ to XYT) or vice versa. These functions can be found in the TOOLS.

Hint: If you have a data stack that is poorly sampled in z (not fulfilling the Nyquist criterion) you better interpret the different planes as independent (i.e. as 2D images) and do 2D deconvolution planewise while taking the optimal Nyquist criterion for z as imposed by the optical parameters (see the diagram in , page). To do this in one run for all the planes, convert the 3D stack to a 2D-time series, do the deconvolution run, and convert back from 2D-time to 3D.

Time series

A time series is a sequence of images recorded along time at uniform time intervals. Every recorded image is a time frame. The Huygens Essential is capable of automatic deconvolution of 2D-time or 3D-time data. There are some tools that are intended only for time series, as the confocal bleaching corrector or the z-drift corrector (page 16).

Adapting the image

In the TOOLS menu you can find a contrast inverter helpful for the processing of brightfield images, see below.

A CROP tool is also available, but its use is recommended only after properly tuning the image parameters and will be explained in a later stage.

In the TOOLS menu you can also find a MIRROR ALONG Z tool, to flip the image when the coverslip is in the top. This is specially important in case of a refractive index mismatch: see *Spherical aberration correction* on page 40.

Processing brightfield images

Brightfield imaging is not a 'linear imaging' process. In a linear imaging process the image formation can be described as the linear convolution of the object distribution and the point spread function (PSF, see page 14), hence the name deconvolution for the reverse process. So in principle one cannot apply deconvolution based on linear imaging to non linear imaging modes like brightfield and reflection. One could say the image formation in these cases IS linear because it is governed by linear superposition of amplitudes. However, microscopes do not measure light amplitudes but rather intensities, the absolute squared values of the amplitudes. Taking the absolute square destroys all phase information one would need to effectively apply deconvolution. Fortunately, in the brightfield case the detected light is to a significant degree incoherent. Because in that case there are few phase relations the image formation is largely governed by the addition of intensities, especially if one is dealing with a high contrast image.

In practice one goes about deconvolving brightfield images by inverting them (using TOOLS > INVERT IMAGE) and processing them further as incoherent fluorescence widefield images. Still, one should watch out sharply for interference like patterns (periodic rings and fringes around objects) in the measured image. As a rule these become pronounced in low contrast images. After the deconvolution run you may reverse to the original contrast setting.

Setting the image channel colors

The Color picker tool (TOOLS > SET CHANNEL COLORS) allows you to alter the colors for the different image channels (see *Multi channel images* on page 17). The color of a particular channel can be edited by clicking the corresponding button. This opens a platform specific color editor (Figure 8).

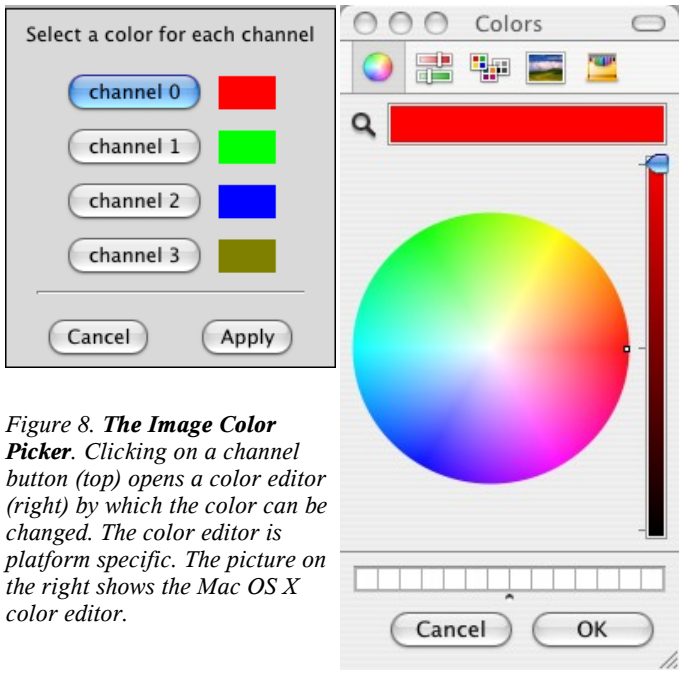


Figure 8. ***The Image Color Picker****. Clicking on a channel button (top) opens a color editor (right) by which the color can be changed. The color editor is platform specific. The picture on the right shows the Mac OS X color editor.*

Image statistics

Right-mouse-click on a thumbnail image and select SHOW PARAMETERS and you will find, besides the parameter settings, statistical information of the particular image. Amongst them are the mean, sum, standard deviation, norm, and position of the center of mass.

Verifying the microscopic parameters

Next to the basic voxel data the Huygens Essential also tries to read as much as possible information about the microscopic recording conditions. However, depending on the file type this information may be incomplete or incorrect. In this first stage all parameters relevant for deconvolution are displayed and can be modified:

Optical parameters (first page):

- Microscope type
- Lens and medium refractive index
- Numerical Aperture (*NA*)

Optical parameters (second page):

- Backprojected pinhole radius in nm. 'Backprojected' means the size of the pinhole as it appears in the specimen plane, see *Computing the backprojected pinhole radius* on page 33.
- Backprojected distance between the pinholes in microns (only visible if the microscope type is 'Nipkow').
- Excitation and emission wavelengths
- Photon count (number of excitation photons involved in the fluorescence)
- Voxel sizes in the three directions *x*, *y*, *z* (third page)
- Summary of all parameters now in effect

If values are displayed in a red background, they are highly suspicious. An orange background indicates a non-optimal situation. Oversampling is also indicated with a cyan background, that becomes violet when it is very severe. Figure 9 shows settings which do not fulfill the criterion for the critical sampling distance versus numerical aperture. (See *Sampling densities* on page 32).

You can see and correct the image parameters not only at this deconvolution stage, but also at any time by right-clicking on the image thumbnail and selecting SHOW PARAMETERS or CORRECT PARAMETERS. Correcting will pop up the window shown on Figure 11.

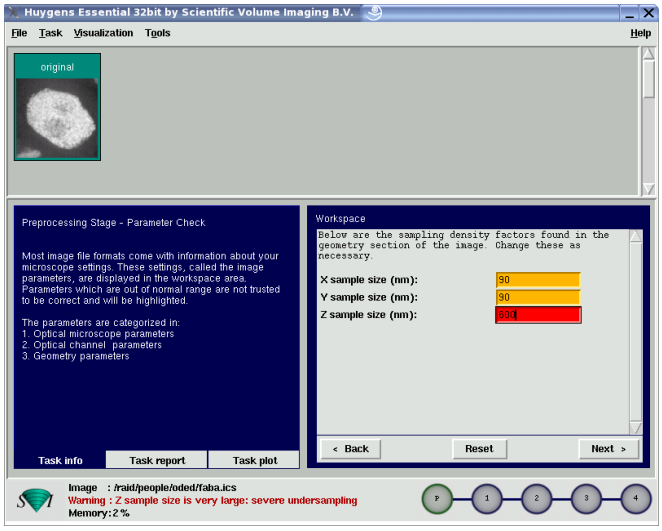

Huygens Essential 32bit by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Preprocessing Stage - Parameter Check
Most image file formats come with information about your microscope settings. These settings, called the image parameters, are displayed in the workspace area. Parameters which are out of normal range are not trusted to be correct and will be highlighted.
The parameters are categorized in:
1. Optical microscope parameters
2. Optical channel parameters
3. Geometry parameters
Task info
Task report
Task plot
Workspace
Below are the sampling density factors found in the geometry section of the image. Change these as necessary.
X sample size (nm): 90
Y sample size (nm): 90
Z sample size (nm): 600
< Back
Reset
Next >
Image : /raid/people/oded/faba.ics
Warning : Z sample size is very large: severe undersampling
Memory: 2 %

Figure 9. ***Parameters check stage: Sampling. Red coloring indicates a suspicious value, and orange a non-optimal value.***

Parameters Templates

Once the proper parameters have been set and verified, they can be saved to a Huygens template file (suffix `.hgst`). Those template files are loadable in the very start of the wizard, hence the user can skip the parameters verification stage, provided that an image is to be restored with the same optical properties as the ones which were recorded on the template.

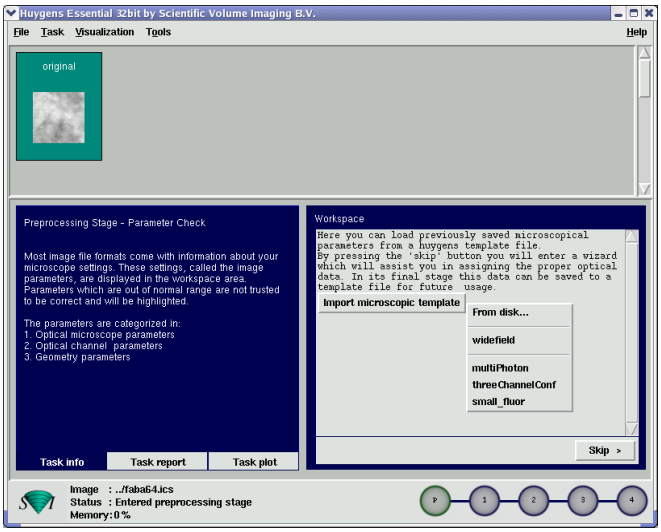

Huygens Essential 32bit by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Preprocessing Stage - Parameter Check
Most image file formats come with information about your microscope settings. These settings, called the image parameters, are displayed in the workspace area. Parameters which are out of normal range are not trusted to be correct and will be highlighted.
The parameters are categorized in:
1. Optical microscope parameters
2. Optical channel parameters
3. Geometry parameters
Task info
Task report
Task plot
Workspace
Here you can load previously saved microscopical parameters from a huygens template file.
By pressing the 'skip' button you will enter a wizard which will assist you in assigning the proper optical data. In its final stage this data can be saved to a template file for future usage.
Import microscopic template
From disk...
widefield
multiPhoton
threeChannelConf
small_fluor
Skip >
Image : ../faba64.ics
Status : Entered preprocessing stage
Memory: 0 %

Figure 10. ***Importing a microscopical parameters template.***

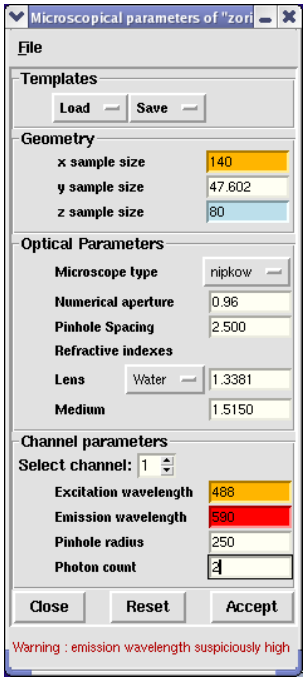


Figure 11. ***Microscopical parameters corrector.***

The IMPORT MICROSCOPIC TEMPLATE button will allow you to choose a template from a list of pre-saved template files which reside both in the common templates directory and in the user's personal template directory. The Huygens common templates directory is named `Templates`, and resides in the Huygens installation directory, namely `/usr/local/svi/Templates` on Unix systems, `C:\Program Files\SVI\Templates` on Windows and `/Applications/SVI/Templates` on the Mac OS X. The user's personal templates directory is called `.svi` on the Unix platforms and `SVI` on Windows, and it can be found in the user's home directory on Unix, and in `C:\Documents and Settings\user_name` on Windows. You can also choose to load a template file from a different location by pressing the FROM DISK... option.

The EXPORT TEMPLATE button will allow you to either save the template to the personal template directory by choosing the TO DISK... option, or overwrite one of the existing templates by selecting them from the list.

The Huygens template is a simple `.xml` file which can be edited 'by hand' as well (see examples in the common `Templates` directory).

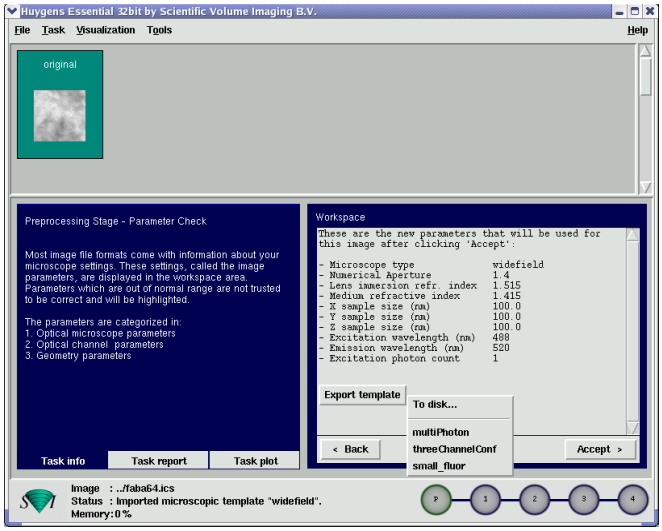

Huygens Essential 32bit by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Preprocessing Stage - Parameter Check
Most image file formats come with information about your microscope settings. These settings, called the image parameters, are displayed in the workspace area. Parameters which are out of normal range are not trusted to be correct and will be highlighted.
The parameters are categorized in:
1. Optical microscope parameters
2. Optical channel parameters
3. Geometry parameters
Task info
Task report
Task plot
Workspace
These are the new parameters that will be used for this image after clicking 'Accept':
- Microscope type widefield
- Numerical Aperture 1.4
- Lens immersion refr. index 1.515
- Medium refractive index 1.415
- X sample size (nm) 100.0
- Y sample size (nm) 100.0
- Z sample size (nm) 100.0
- Excitation wavelength (nm) 488
- Emission wavelength (nm) 520
- Excitation photon count 1
Export template
To disk...
multiPhoton
threeChannelConf
small_fluor
< Back
Accept >
Image : ../faba64.ics
Status : Imported microscopic template "widefield".
Memory: 0 %

Figure 12. ***Exporting a microscopical parameters template.***

The intelligent cropper

The time needed to deconvolve an image increases more than proportional with its volume. Therefore, deconvolution can be accelerated considerably by *cropping* the image. Huygens Essential is equipped with an intelligent cropper which automatically surveys the image to find a reasonable proposal for the crop region. In computing this initial proposal the microscopical parameters are taken into account, making sure that cropping will not have a negative impact on the deconvolution result. Because the survey depends on accurate microscopical parameters it is recommended to use the intelligent cropper as final step in the preprocessing stage, but you can launch it before the restoration process from the TOOLS > CROP ORIGINAL IMAGE menu. Once you have cropped your image during the guided restoration process you can not crop it again except after closing the image and reloading it again.

Cropping an image in *x y z*

After you have verified your image parameters the Crop tool is launched if you press the YES button to the question 'Launch the cropper?'. The cropper will look as in Figure 13 (the image will be in gray scale mode if it is a single channel). Red lines indicate the borders of the proposed cropping region. This is computed from the image content and the microscopic parameters at launch time of the cropper.

The cropper allows manual adjustment of the proposed crop region. To adjust the crop region put the cursor *inside* the red boundary, press the left mouse button and keep it pressed to sweep out a volume. Accept the new borders by pressing the CROP button. Do not crop the object too tightly, because you would remove blur information relevant for deconvolution. Do not crop the image to make it too large along the optical axis Z, an aspect ratio close to 1:1:1 (or less than 1 for Z) is much better.

The three views shown are Maximum Intensity Projections (MIP's) along the main axes. The projections are computed by tracing parallel rays perpendicular to the projection plane through the data volume, each ray ending in a pixel of the projection image. The maximum intensity value found in each ray path is projected. For example, each pixel in the *xy* projection image corresponds with the maximum value in the vertical column of voxels above it.

By default the projections are over the whole dataset (including all the frames in time series), but this might be confusing sometimes. The small colored triangles can be used to restrict the projections within a specific range of slices.

Cropping an image in time

You also can reduce the number of time frames by selecting TIME > SELECT FRAMES from the CROP menu, as shown in Figure 14. This applies to time series (see *Time series* on page 8).

Removing channels

You can remove channels from a multi-channel image (see p. 17) using the crop tool's channels selecting tool CHANNELS > SELECT CHANNELS.

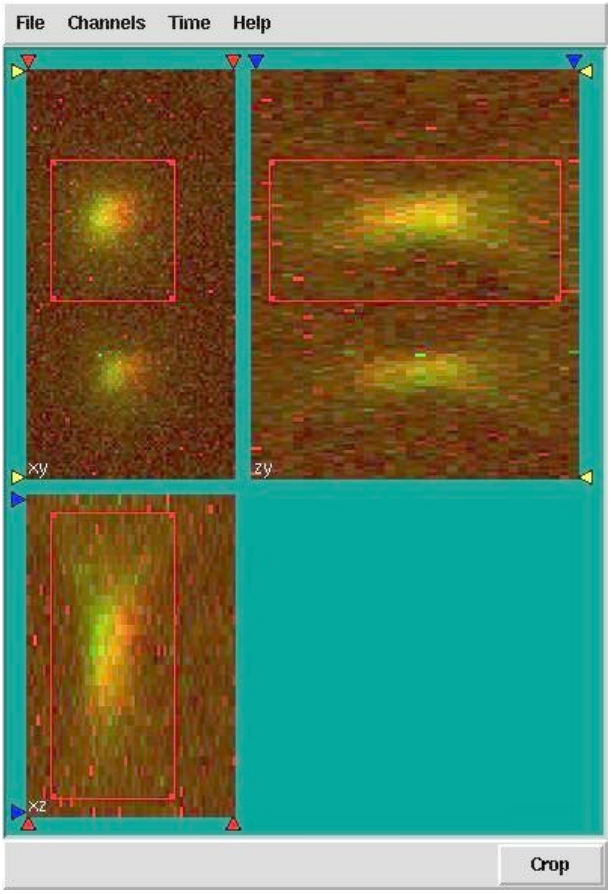


Figure 13. ***The Crop tool.***

Stage 1: Parameter tuning

Stage 1 enables you to tune your parameter settings as set in the pre-processing stage. After you have finish cropping stage 1 is skipped and you will directly jump into the image histogram (stage 2). Still you may wish to tune your parameter settings afterwards. You can enter stage 1 by pressing the RESTART button in the latest stage.

Stage 2: The image histogram

The next stage shows you the image histogram.

The histogram image is an important statistical tool for *inspecting* your image. It is included to let you spot problems that might have occurred during the recording. It has no image manipulation options as such, it just may prevent you from future recording problems.

The histogram shows the number of pixels as a function of the intensity (gray value) or groups of intensities. If your image is an 8-bit image (gray values from 0−255) the x-axis is the gray value and the y-axis is the number of pixels in the image with that gray value. If the image is more than 8-bits gray values are collected to form a 'bin' (for example gray values from 0−9 form bin 0, values from 10−19 form bin 1, etc.) The histogram is now the number of pixels in every bin.

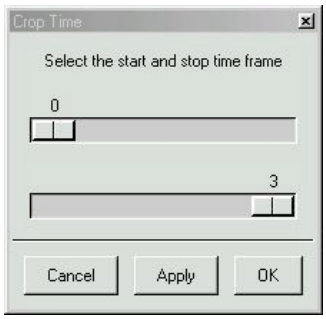


Figure 14. ***Reducing the number of time frames.***

The histogram from the demo image as shown in Figure 15 is of reasonable quality. The narrow peak you see at the left represents the background pixels, all with similar values. The height of the peak represents the amount of background pixels. Because in this particular image there are many voxels with a value in a narrow range around the background the peak is higher than the other.

In this case there is also a small black gap at the left of the histogram. This signifies an electronic offset (blacklevel) in the signal recording chain of the microscope (see page 32).

If a peak is visible at the extreme right hand side of the histogram it indicates saturation or 'clipping'. Clipping is caused by intensities above the maximum digital value available in your microscope. Usually, all values above the maximum value are replaced by the maximum value. On rare occasions they are replaced by zeros. Clipping will have a negative effect on the results of deconvolution, especially with WF images. (See page 38).

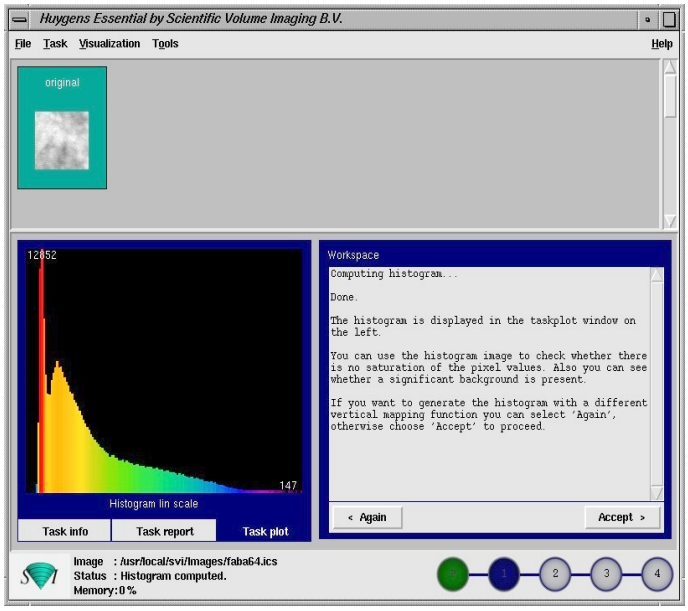

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
12852
147
Histogram lin scale
Task info
Task report
Task plot
Workspace
Computing histogram...
Done.
The histogram is displayed in the taskplot window on the left.
You can use the histogram image to check whether there is no saturation of the pixel values. Also you can see whether a significant background is present.
If you want to generate the histogram with a different vertical mapping function you can select 'Again', otherwise choose 'Accept' to proceed.
< Again
Accept >
Image : /usr/local/svi/Images/faba64.ics
Status : Histogram computed.
Memory: 0 %

Figure 15. ***The image histogram.*** *The vertical mapping mode can be selected from linear, logarithmic or sigmoid.*

The histogram stage is included for examining purpose only. It has no meaning for the deconvolution process that follows[3].

Stage 3: Estimate the average background in the image

In this stage the *average* background in a volume image is estimated. The average background is thought to correspond with the noise-free equivalent of the background in the measured (noisy) image. It is determined by searching the image first for a region with low values. Subsequently the value for the background is determined by searching in this region for the area with radius *r* which has the lowest average value. It is important for the search strategy that the microscopic parameters of the image are correct, in especially the sampling distance and the microscope type.

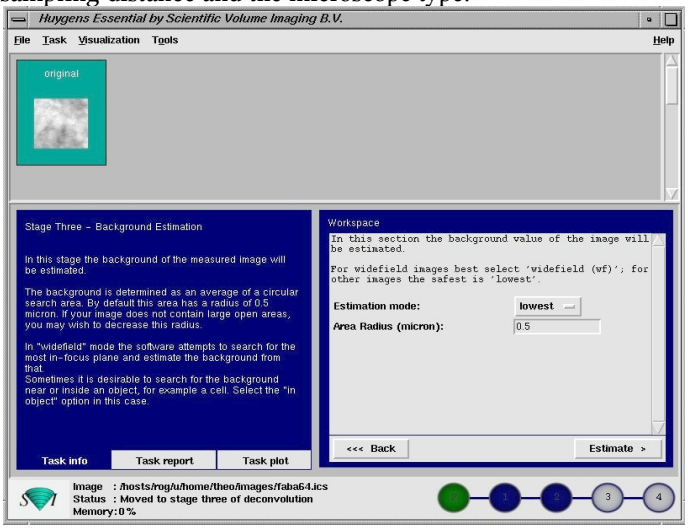

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Stage Three - Background Estimation
In this stage the background of the measured image will be estimated.
The background is determined as an average of a circular search area. By default this area has a radius of 0.5 micron. If your image does not contain large open areas, you may wish to decrease this radius.
In "widefield" mode the software attempts to search for the most in-focus plane and estimate the background from that.
Sometimes it is desirable to search for the background near or inside an object, for example a cell. Select the "in object" option in this case.
Task info
Task report
Task plot
Workspace
In this section the background value of the image will be estimated.
For widefield images best select 'widefield (wf)'; for other images the safest is 'lowest'.
Estimation mode:
lowest
Area Radius (micron):
0.5
<<< Back
Estimate >
Image : /hosts/rog/u/home/theo/images/faba64.ics
Status : Moved to stage three of deconvolution
Memory: 0 %

Figure 16. ***Estimating the average background.***

The following choices are possible here:

- *Lowest value* (Default): The image is searched for a 3D region with the lowest average value. The axial size of the region is around 0.3 micron; the lateral size is controlled by the *radius* parameter which is default set to 0.5 micron.
- *In/near object*: The neighborhood around the voxel with the highest value is searched for a planar region with the lowest average value. The size of the region is controlled by the *radius* parameter.
- *WF*: First the image is searched for a 3D region with the lowest values to ensure that the region with the least amount of blur contributions is found. Subsequently the background

3 Learn more about histograms in `http://support.svi.nl/wiki/ImageHistogram`

is determined by searching this region for the planar region with radius r that has the lowest value.

Press the ESTIMATE button to continue. You may now adapt the value if you like to, either by altering the value in the ESTIMATE BACKGROUND field or in the RELATIVE BACKGROUND field. Setting this last to -10, for example, lowers the estimated background with 10%. If you are done press ACCEPT to start the last stage, the true deconvolution process.

Stage 4: Deconvolution

Before starting the actual iterative deconvolution run, stage 4 first carries out several preprocessing steps:

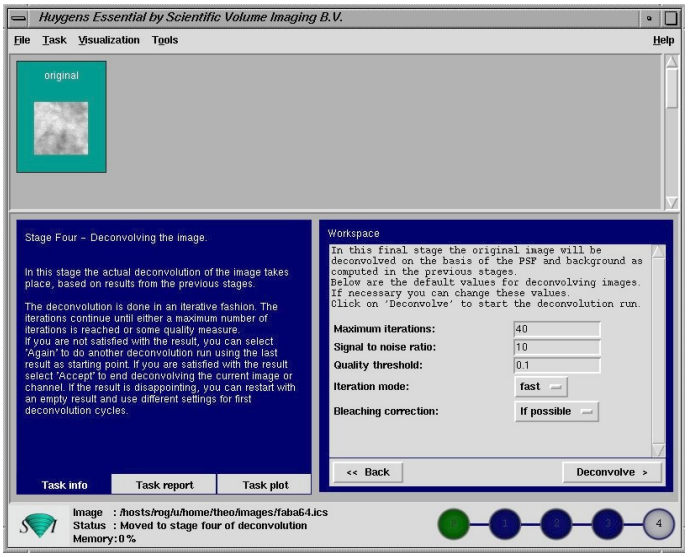

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Stage Four – Deconvolving the image.
In this stage the actual deconvolution of the image takes place, based on results from the previous stages.
The deconvolution is done in an iterative fashion. The iterations continue until either a maximum number of iterations is reached or some quality measure.
If you are not satisfied with the result, you can select "Again" to do another deconvolution run using the last result as starting point. If you are satisfied with the result select "Accept" to end deconvolving the current image or channel. If the result is disappointing, you can restart with an empty result and use different settings for first deconvolution cycles.
Task info
Task report
Task plot
Workspace
In this final stage the original image will be deconvolved on the basis of the PSF and background as computed in the previous stages.
Below are the default values for deconvolving images.
If necessary you can change these values.
Click on 'Deconvolve' to start the deconvolution run.
Maximum iterations: 40
Signal to noise ratio: 10
Quality threshold: 0.1
Iteration mode: fast
Bleaching correction: If possible
<< Back
Deconvolve >
Image : /hosts/rog/u/home/theo/images/faba64.ics
Status : Moved to stage four of deconvolution
Memory: 0 %
4

Figure 17. ***Stage 4: starting the deconvolution.***

1. Background: This value is calculated in stage 3. You can verify whether this value represents areas in the image which you consider background by opening the Twin Slicer (see Figure 20) and moving the mouse pointer over the areas of interest. The voxel values are displayed below the image. Modify the value as you see fit.
2. A Point Spread Function (PSF, see below) was generated from the established microscopic parameters. This took place off the screen and is fully transparent to the user.
3. If the size of the computer's RAM is too small to deconvolve the image as a whole, it is split up in parts called 'bricks'. SVI's Fast Classic Maximum Likelihood Estimation (MLE) algorithm runs on the image or on all the bricks and fits the deconvolved bricks seamlessly together (see *Brick wise processing* on page 30).

The Point Spread Function (PSF)

One of the basic concepts in image deconvolution is the Point Spread Function. The PSF of your microscope is the image which results from imaging a point object in the microscope. Due to wave diffraction a point object is imaged, 'spread out', into a fuzzy spot: the PSF. In fluorescence imaging the PSF completely determines the image formation. In other words: all microscopic imaging properties are packed into this 3D function. The PSF can be obtained in two different ways:

1. Generating a theoretical PSF: When a measured PSF is not available, Huygens Essential automatically uses a theoretical PSF. The PSF is computed from the microscopic parameters that come with your image and which you have double-checked in the preprocessing 'P' stage or in stage one. Because a theoretical PSF can be generated without any user intervention Huygens Essential does the calculation in the background without any notice.

Images affected by spherical aberration (S.A.) due to a refractive index mismatch are better restored through the use of theoretical depth-dependent PSF's. Read about S.A. on page 38, and how to correct it on page 40.

2. Measuring a PSF: By using the PSF Distiller or the tools in Huygens Professional you can derive a measured PSF from images of small (< 200 nm) fluorescent beads.
You can load a previously measured PSF with FILE > OPEN PSF... (Main menu). If you load a PSF, Huygens Essential will automatically use it. If the measured PSF contains less channels than the image, a theoretical PSF will be generated for the channels where there is no PSF available.
See *The PSF Distiller* on page 27 for more information on measuring a PSF.

A measured PSF should only be used for deconvolution if the image and the bead(s) were recorded with the same microscope at the same parameter settings as the bead image(s).

The Classical Maximum Likelihood Estimation (CMLE) algorithm

Huygens Essential uses the Classical Maximum Likelihood Estimation for the deconvolution process. This method is an extremely versatile algorithm, applicable for all types of data sets[4].

The following option values may be set:

Number of iterations

MLE-based deconvolution uses an iterative process that never stops if no stopping criterion is given. This stopping criterion can simply be a maximum number at which the process will stop. This value depends on the desired final quality of your image. For an initial run you can leave the value at its default. To achieve the best result you can increase this value.
Another stopping criterion is one based on the Quality change of the process, see *Quality threshold* below.

Signal to noise ratio

You have to make an estimation of the SNR from your recorded image. Inspect your image and decide if your image is noisy (SNR < 10), has moderate noise (10 < SNR < 20) or is a low-noise image (SNR > 20). See *Signal to Noise Ratio (SNR)* on page 31, and some examples of noisy images in Figure 29.

Quality threshold

Beyond a certain amount of iterations, typically below 100, the difference between successive iterations becomes insignificant and progress grinds to a halt. Therefore it is a good idea to monitor progress with a quality measure, and to stop iterations when the change in quality drops below a threshold. At a high setting of this quality threshold (e.g. 0.1) the quality difference between subsequent iterations may drop below the threshold before the indicated maximum number of iterations has been completed. The smaller the threshold the larger the number of iterations which are completed and the higher the quality of restoration. Still, the extra quality gain becomes very small at higher iteration counts.

4 Huygens Professional also has Quick-MLE-time, Quick-Tikhonov-Miller, and Iterative Constrained Tikhonov-Miller.

Iteration mode

In FAST MODE (highly recommended) the iteration steps are bigger than in HIGH QUALITY mode. More information can be read in the DICTIONARY from the HELP menu.

Bleaching correction

The data is inspected for bleaching. 3D images and time series of WF images will always be corrected. Confocal images can only be corrected if they are part of a time series, and when the bleaching over time shows exponential behavior.

Stopping the MLE algorithm

Pressing RESTORE starts the iterative MLE algorithm; a STOP button appears. Pressing STOP halts the iterations and retrieves the result from the previous iteration. If the first iteration is not yet complete a empty image will result.

Finishing or restarting a deconvolution run

When a deconvolution run is finished use the *Twin Slicer* (page 18) to inspect the result in detail. Depending on the outcome of that you can choose AGAIN, RESUME or ACCEPT:

AGAIN discards the present result, and re-runs the deconvolution, possibly with different parameters.

RESUME re-runs the MLE procedure without discarding the result, and with the possibility to change the deconvolution parameters. The software will ask you to continue were you left off (keeping improving the image, quite recommendable) or to start from the raw image again. A new result will be generated to compare with the previous one, for instance using the Twin Slicer. You can repeat this several times.

ACCEPT proceeds to the final stage or, if the data was multi-channel, to the next channel (see page 17). If you generated several results by resuming the deconvolution you will be asked to select the best result as the final one, that will be renamed to 'deconvolved'. The other results will remain as well in case you want to save them.

z-drift corrector for time series

For 3D time series the program pops-up an additional tool that enables you to correct for movement in the *z* (axial) direction that could have been occurred for instance by thermal drift of the microscope table. In case of a multi channel image (see p. 17), the corrector can survey ALL CHANNELS and determine the mean *z* position of the channels, or it can take ONE CHANNEL as set by the REFERENCE CHANNEL parameter.

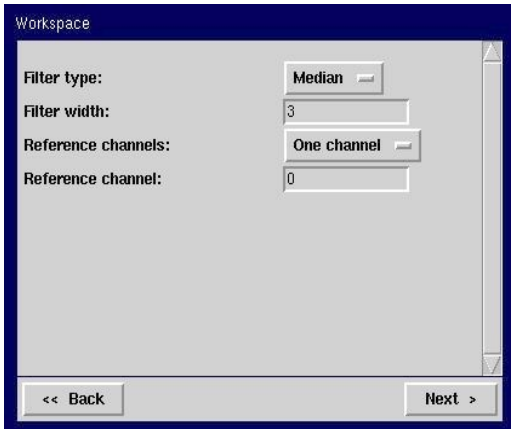


Figure 18. ***The z-drift corrector.***

After determining the *z* positions per frame, the *z*-positions can be filtered with a MEDIAN, GAUSSIAN or KUWAHARA filter of variable width. When the drift is gradual, a gaussian filter is probably best. In case of a drift with sudden reversals or outliers a median filter is best. In case the *z* positions show sudden jumps, we recommend the edge preserving Kuwahara filter.

Saving the result

Saving the restored image

After each deconvolution run you can save the result. Select the image to be saved and do FILE > SAVE '*IMAGENAME*' AS... in the menu bar. You can save the image as an Image Cytometry Standard (ICS or ICS2) image file, a TIFF file series, an Imaris-Classic file or a Biorad `.pic` file. Only the ICS and ICS2 file type save all the microscopic parameters. For information on how to proceed with multi-channel data see *Joining the results* on page 18.

The ICS file format actually uses two separate files: a header file with `.ics` extension and other, much bigger and with the actual image data, with `.ids` extension. On the other hand the newer ICS2 file format uses only one single `.ics` file with both the header and the data together.

Saving your Task report

Select from the Main menu bar TASK > SAVE TASK REPORT to store the information as displayed in your *Task report* tab deck.

Multi channel images

Fluorescent microscopes can usually register different emission wavelengths (almost) simultaneously, allowing you to image different dyes on the sample. In the terminology of the Huygens Software, one channel in an image refers to the intensity distribution recorded at a given fixed wavelength, independently of what device made the acquisition. Thus, it is a logical channel of stored data, and not necessarily a physical channel (as all the image channels could have been measured by a single photomultiplier, for instance).

Multi channel images can be deconvolved in a semi automatic fashion, giving you the opportunity to fine tune the results obtained with each individual channel. *After the preprocessing stage* the multi channel image is split into single channel images named 'channel-0', 'channel-1', and so on. The first of these is automatically selected for deconvolution. To deconvolve it, proceed as follows:

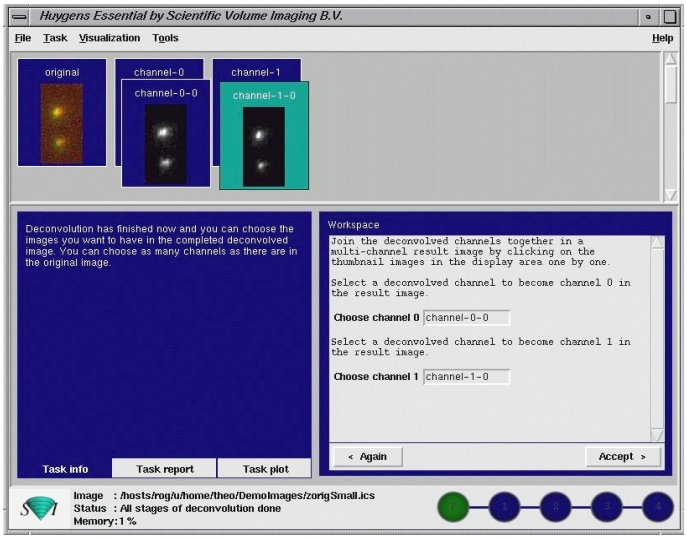

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
channel-0
channel-0-0
channel-1
channel-1-0
Deconvolution has finished now and you can choose the images you want to have in the completed deconvolved image. You can choose as many channels as there are in the original image.
Workspace
Join the deconvolved channels together in a multi-channel result image by clicking on the thumbnail images in the display area one by one.
Select a deconvolved channel to become channel 0 in the result image.
Choose channel 0 channel-0-0
Select a deconvolved channel to become channel 1 in the result image.
Choose channel 1 channel-1-0
Task info
Task report
Task plot
< Again
Accept >
Image : /hosts/rog/u/home/theo/DemoImages/zorigSmall.ics
Status : All stages of deconvolution done
Memory: 1 %

Figure 19. ***Deconvolving a two channel image.***

Deconvolving a channel in a multi channel image

The procedure to deconvolve a channel in a multi channel data set is exactly the same as for a single channel image. You can therefore do multiple reruns on the channel at hand, just as you can with single channel data. When you are done press ACCEPT in the last (stage 4) screen. This will cause the next channel to be selected for restoration. Proceed as usual with that channel and the remaining channels. If you do not want to process all the channels in an image you may skip one or more channels.

Joining the results

When you press ACCEPT for the last channel you enter a screen which allows you to select the results which you want to combine into the final deconvolved multi channel image. This means that up to this point you can still change your mind as to which of the results you want to combine, even in what order. Once you press ACCEPT a multi channel image named *Restored* is created. To save it go to the FILE > SAVE 'RESTORED' AS menu.

4. Huygens Essential Visualization Tools

Huygens Essential provides different tools for data visualization.

The Twin Slicer

This allows you to compare a deconvolution result with the original, but also different deconvolution results obtained from the same original.

Open the Twin Slicer by double clicking on one of the thumbnail images in the main window (or by right-clicking on the image thumbnail, then SHOW ON TWIN SLICER). The Twin Slicer will show the selected image on the left. By clicking on the menu bar below the image you can select a different image. Likewise, the bar below the right display field gives access to one of the other images currently present in Huygens Essential (see Figure 20). Currently only two images with the same dimensions can be displayed at the same time.

If you select the same image twice, you may compare different slices from the same image. For this, first move the slider until the desired position, then click on one of the image's name to DISABLE the action of the slider on it: the slider will then affect only the other image view (see Figure 21).

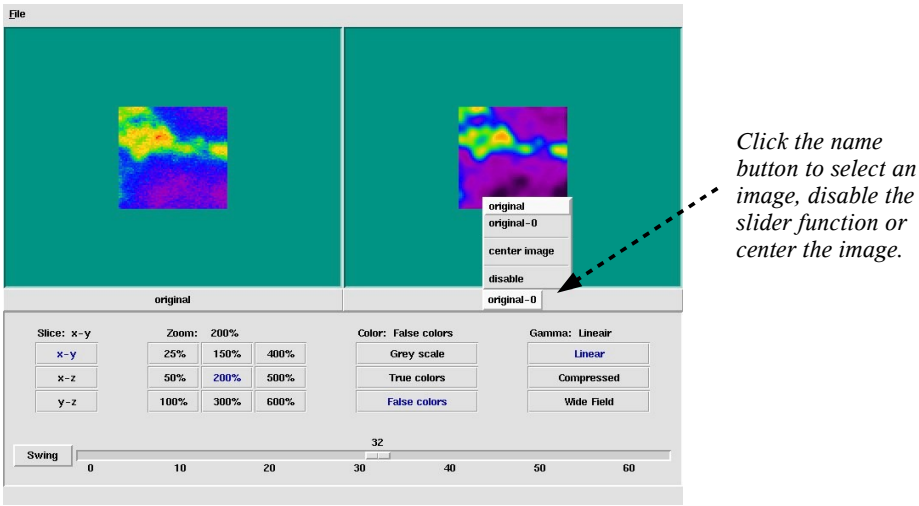


Figure 20. ***The Twin Slicer****, applied to a 3D dataset. The selected display settings are highlighted in blue.*

Pixel intensity values for the cursor position on the image are displayed at the bottom of the window. You can move the image by clicking the left-mouse button and keeping it pressed while moving the image to the desired position. You can center the image again by selecting CENTER IMAGE from the name button.

With the slider you can slice your images along the three axis, depending on what is the selected slice view. You can also change the zoom factor, the color mode and the contrast mapping mode.

Color mode

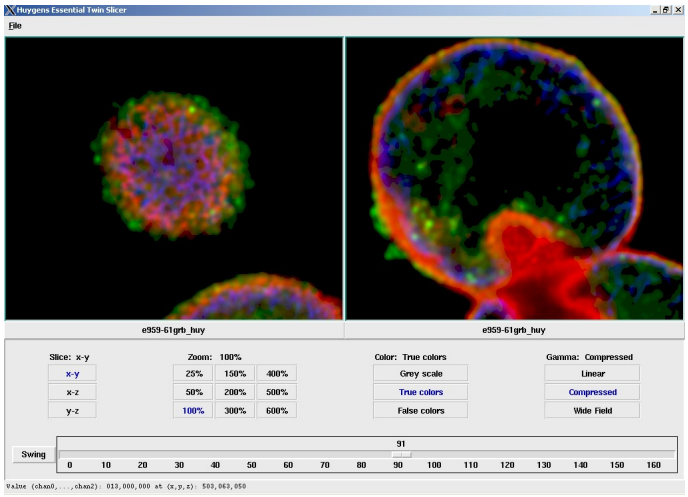


Figure 21. The Twin Slicer showing two slices of the same 3D image.

- Gray: pixel values are assigned different shades of gray ranging from black for the lowest values to white for the highest values.
- False color: pixels values are assigned different colors ranging from black/dark purple for the lowest values to bright red for the highest value.
- True color: pixel values are assigned different shades of a particular color ranging from black for the lowest values to the brightest possible shade for the highest values.

Multi channel images are always rendered with a true color scheme, otherwise the information from the different channels will result in very confusing images.

Contrast mapping mode

- Linear (default): In this mode the pixel values are mapped to screen buffer color intensities in a linear fashion. Note that the actual translation of the screen buffer values to the actual brightness of a screen pixel is usually quite non-linear.
- Compress: Where an image contains a few very bright spots and some larger darker structures using Linear mode will result in poor visibility of the darker structures. Restoration of such images is likely to further increase the dynamic range resulting in the large structures becoming even dimmer. In such cases use the compress display mode to increase the contrast of the low valued regions and reduce the contrast of the high-valued regions. Another way to improve the visibility of dark structures is the usage of false colors (see above).
- Widefield (WF) mode: In restoring widefield images it sometimes happens that blur removal is not perfect, for instance when one is forced to use a theoretical point spread function in sub optimal optical conditions. In such cases the visibility of blur remnants can be effectively suppressed.

Time series

If you open the Twin Slicer on a time series a second slider is added. Both the time-slider and the spatial-slider have a swing option. When the spatial swing is pressed the slider moves back and forth; when the time swing button is pressed the slider only moves forward, i.e. in the positive time direction.

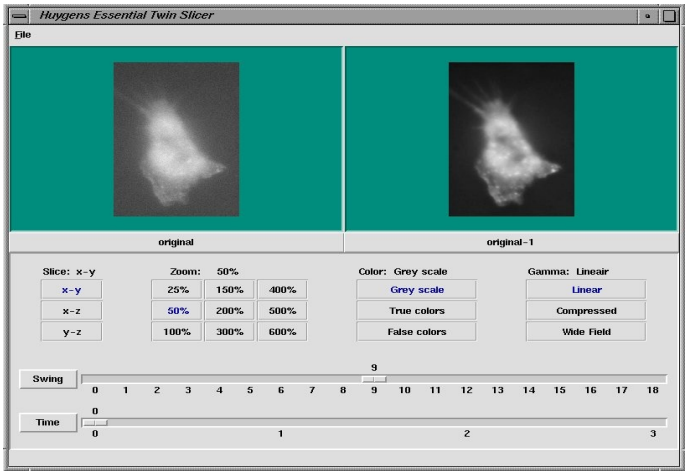


Figure 22. ***The Twin Slicer with time slider.***

The MIP Renderer

The Maximum Intensity Projection (MIP) Renderer is part of the Huygens Essential since release 2.6.0p4 and enables you to obtain a spatial projection of your data from a given viewpoint (see Figure 23).

The renderer projects, in the visualization plane, the voxels with maximum intensity that fall in the way of parallel rays traced from the viewpoint to the plane of projection. Notice that this implies that two MIP renderings from opposite viewpoints show symmetrical images.

To start the MIP Renderer, right-click on an image's thumbnail to open the contextual menu, then select SHOW IN MIP RENDERER.

Select your viewpoint by moving the 'Tilt' and 'Twist' sliders, or by dragging the mouse pointer on the large view (that will be empty at first, before your first rendering). Also try changing the zoom. You will see how the preview thumbnail changes. When you have set all the rendering options, click Render to create the final view, that you can save as a TIFF image.

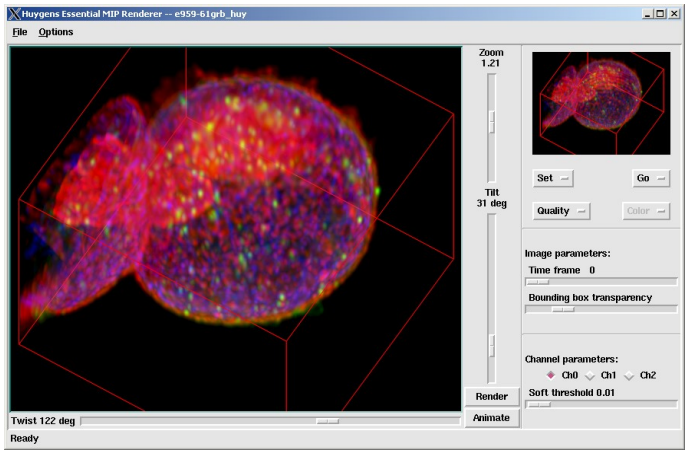


Figure 23. ***The Maximum Intensity Projection (MIP) Renderer***

You will find different rendering options on the window, and also in the Options menu. The configurable parameters are the rendering size and quality, the appearance of the bounding box, and the mode of the soft thresholds applied to the image channels.

Soft threshold

A soft threshold is a preprocessing tool that reduces the background in the image, so voxels with intensity values below the threshold value become more transparent. Contrary to a standard threshold, that is 'all or nothing' (values above the threshold are kept, values below it are deleted), the soft threshold function handles images in a different way. It makes smooth transitions between the original an the deleted values. If the original value S of a voxel is $S >$ (threshold value + range/2) then the final filtered value D does not change ($D = S$). If $S <$ (threshold value - range/2)

then the voxel is 'deleted' ($D = 0$). For the values in between, a smooth function is applied: if (threshold value -range/2) < S < (threshold value + range/2) then $D = f(S)$ according to a shape function, which in this case is a sinusoidal. By changing the parameters in Options > Soft threshold mode from Hard to Soft, you progressively increase the 'range' value, thus broadening the transition from the original to the deleted values.

You can apply different soft thresholds to the different image channels.

Rendering a movie

With the MIP Renderer you can also make an animation of your image, changing the viewpoint in different frames. Select the viewpoint coordinates for the first frame, then click Set > Home. Select now the viewpoint coordinates for the last frame, and click Set > End. (You can now go to the last or the first frame by clicking Go > End or Go > Home). Select all the rendering parameters, including the total number of rendered frames for the movie (Options > Animation frame count). Finally, click Animate, and select a directory to save the TIFF frames to. You can later load and edit these TIFF images with your favorite animation tool. For instance, you can use the `convert` tool from ImageMagick (`http://www.imagemagick.org`) to make a GIF animation, using

```
convert -delay 20 animatedMip*.tiff animatedMip.gif
```

You can now place this single file GIF animation directly on your web page, as most of the Internet browsers currently available can handle this kind of movie files.

With the appropriate codec, you can also use `convert` to make a MPEG animation. See the ImageMagick website for more details.

If your image is a time series, you can also make an animation along time frames.

The SFP Renderer

Starting from Essential 2.5, a simplified version of SVI's high end volume renderer FluVR (Fluorescence Volume Renderer) is available for visualizing your volumetric object from a selectable viewpoint. Like FluVR, this renderer is based on taking the volume image as a distribution of fluorescent material, simulating what happens if the material is excited and how the subsequently emitted light travels to the observer. The computational work is done by the Simulated Fluorescence Process (SFP) algorithm.

The ray-tracing technique does not require a special graphical board as the polygon based techniques do.

To start the SFP renderer, right-click on an image's thumbnail to open the contextual menu, then select Show in SFP renderer (see Figure 24).

Summary

A virtual light source produces excitation light that illuminates the object. This casts shadows either on parts of the object itself or on a table below it. The interaction between the excitation light and the object provokes the emission light, that also interacts with the object before it reaches the eye of the viewer (see Figure 25).

SFP fundamentals

The voxel values in the image are taken as the density of a fluorescent material. If the voxels are multiparameter (“multi-channel” in microscopic parlance, see page 17) each parameter is taken as

a different fluorescent dye. Each dye has its specific excitation and emission wavelength with corresponding distinct absorption properties. The absorption properties can be controlled by the user. The different emission wavelengths give each dye its specific color.

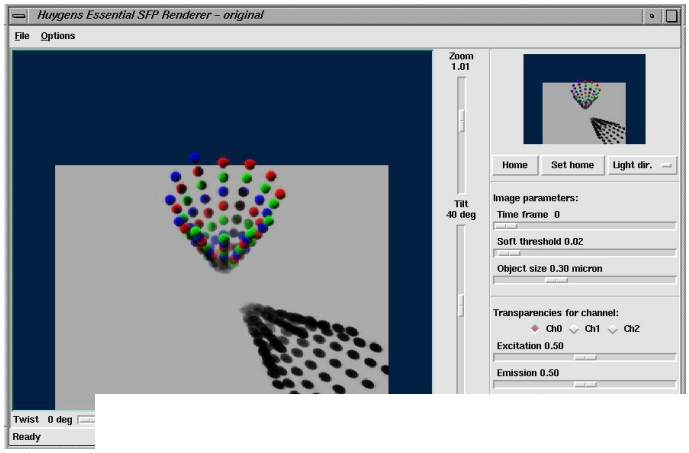


Figure 24 subsampl actual re

To excite the fluorescent matter light must traverse other matter. The resulting attenuation of the excitation light will cause objects, which are hidden from the light source by other objects, to be weakly illuminated, if at all. The attenuation of the excitation light will be visible as shadows on other objects. To optimally use the depth perception cues generated by these shadows a homogeneous plane (the gray table) below the data volume is placed on which the cast shadows become clearly visible.

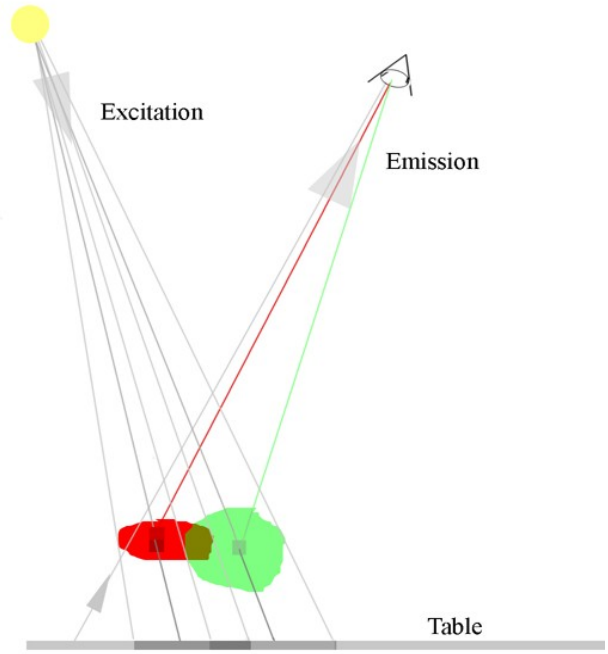


Figure 25. ***With the SFP renderer excitation and subsequent emission of light of fluorescent materials is simulated.*** *Each subsequent voxel in the light beam (excitation) is affected by shadowing from its predecessors. The transparency of the object for the emission light controls to what extent the viewer can peer inside the object.*

The light source is drawn here inside the figure, but in real is placed at infinite distance as to make the light rays parallel.

The renderer in Essential is in non-perspective mode (so called orthogonal projection), i.e. the viewpoint is at infinity.

After excitation the fluorescent matter will emit light at a longer wavelength. Since this emitted light has changed wavelength it is not capable to re-excite the same fluorescent matter: multiple scattering does not occur. Thus only the light emitted in the direction of the viewer, either directly or by way of the semi reflecting table is of importance. By simulating the propagation of the emitted light through the matter, the algorithm computes the final intensities of all wavelengths (the spectrum) of the light reaching the viewpoint. By default the first channel (ch-0) is the red object, the second channel is (ch-1) is the green object and the third (ch-2) is the blue object.

The properties of the interaction between object and light (transparency), both for excitation and emission, as well as the viewpoint, can be adapted interactively by the user to produce different sceneries. Since the volume rendering process is rather compute intensive, a preview image is displayed (see Figure 24). Apart from the viewpoint settings and the optional zooming, the following sliders affect the image:

- Transparencies:

    - Excitation: The transparency of the object for the excitation light. The less transparency the more shadow is casted on the subsequent voxels and on the table.
    - Emission: The transparency of the object for the emission light. The lower the transparency for the emission light the more difficult it is to peer inside or trough the object.
- The Characteristic object size affects both the excitation and the emission transparency. While traveling through the object, the light intensity is attenuated to some degree. This enables us to define some definition for penetration depth at which the light intensity is decreased to some extent, say 10% of its initial value. This penetration depth should be in line with the object size. A transparent object is small with respect to the penetration depth. Thus for the same physical properties of the light one object can be transparent while the other is oblique due to its size. To find a reasonable range in transparencies the object size may be altered. At start-up the object size is computed from the microscopic sampling sizes and number of pixels the image is composed off. If your image has not the correct parameters (for example a TIFF series) the object size is set according to the default parameters as set by the Huygens Essential software and may not be related to the actual object size.
- Frame: Time series (frames) can be handled. For a 3-D image this slider is inactive and set to Frame 0.
- Object brightness: Intensity of the virtual light source.
- Soft threshold: Preprocessing tool that reduces the background in the image. Voxels with gray values below the threshold value become more transparent. See a detailed explanation in the MIP Renderer section, on page 20.

Use the RENDER button to start the actual rendering. The result can be saved as a TIFF-image (FILE > SAVE).

Use LIGHT DIRECTION to alter the direction of the light.

You can open as many SFP windows as you like.

Rendering a movie

With the SFP Renderer you can also make an animation of your image, changing the viewpoint or the time coordinate in different frames. The procedure is analogous to the one explained for the MIP renderer on page 21.

The Surface Renderer

The Surface Renderer is available from the Huygens Essential version 3.0 onwards, and enables you to represent your data in a convenient way to clearly see separated volumes. Because this Surface Renderer is based on fast raytracers, there is no need for any special graphic card as would be necessary for conventional polygon based techniques.

The Surface renderer is an extended optional tool, and is enabled by a v flag in the license string (see *License string details* on page 41).

To start the Surface Renderer, right-click on an image's thumbnail to open the contextual menu, then select SHOW IN SURFACE RENDERER. Let the renderer initialize.

You can find three graphic pipes to redirect your image data channels to: two surface pipes, and one MIP pipe. These can be activated independently.

Use the threshold slider to apply different thresholds to your data channels, to select what voxels are considered to shape volumes. Connected voxels after the threshold determine independent volumes, that will be represented by the 3D surfaces containing them, with different colors. You can use up to two different data channels for surface rendering, one in each of the two available surface graphic pipes. The color of the different objects inside a channel can be modified with a selector (see *Hue Selector* below).

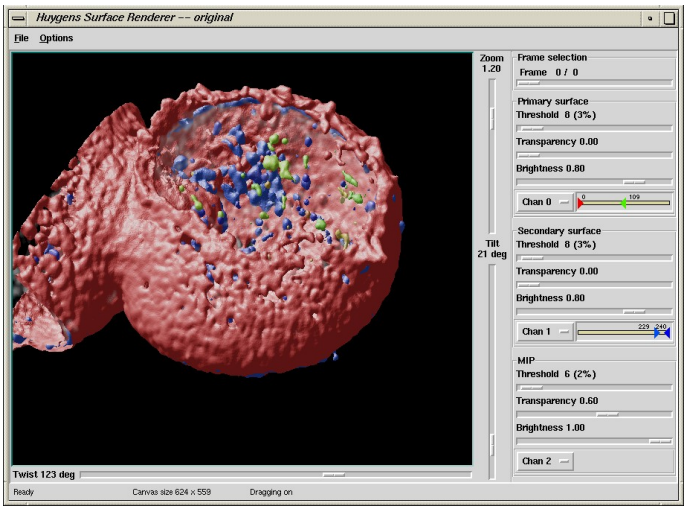


Figure 26. ***The Surface Renderer.***

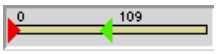
There is a third graphic pipe to redirect data to the rendered image: the MIP pipe works projecting the voxels with maximum intensity laying in the path of the rays traced along the viewing direction (see *The MIP Renderer* on page 20). In combination with the surface pipes, you can obtain very clear representations of the different objects in your image. The MIP rendering of one channel can be a good spatial reference for the objects in the other channels.

You can control the transparency and the brightness of the rendered surfaces with the corresponding sliders, independently in each graphic pipe.

Select the viewpoint by moving the 'Tilt' and 'Twist' sliders, or by dragging the mouse pointer on the large view. Also try changing the zoom. When you have your rendering ready, save it to a TIFF file with FILE > SAVE.

Other available options to change are render size and transparency depth, accessible through the OPTIONS menu.

Hue Selector



The hue selector is a component that allows you to select the color range (actually, the hue property of the color) in which the different objects of each channel are displayed by the Surface Renderer or the Colocalization Analyzer. Thus, objects belonging to different channels can be represented with very different hue ranges to make them clearly distinct, but also with some gradual differences inside the selected range to distinguish independent objects. You can also collapse a range to have all objects in a channel displayed with exactly the same color.

Transparency depth

This option controls how different surfaces are seen through the others. The effects are mainly visible when you have some objects intersecting with other. With the SIMPLE depth, only the piece of surface closest to the viewer's eye screens the others behind it, with its corresponding transparency level.

With the NORMAL depth, up to two pieces of surface are considered to screen other objects. Thus, one object B *inside* the surface A will appear less screened than a third object C *behind* A. B is only screened by the piece of surface A closer to the viewer's eye, while the object C is screened by two pieces of surface A.

The DEEP option will consider many more screening levels, making the final rendering more complex.

5. Huygens Essential Analysis Tools

Huygens Essential is extended with new tools for interactive analysis of 3D and 4D microscopic images. The Colocalization Analyzer is present in Huygens Essential since version 3.0, and an Object Analyzer will be available soon.

The Colocalization Analyzer

The Colocalization Analyzer provides information about the amount of spatial overlap between structures in different data channels, for 3D images and time series.

As this overlapping can be defined in many ways, Huygens gives you the colocalization coefficients most commonly used in literature: Pearson, Overlap, and Manders *M* and *K* (see *Colocalization Theory* in the SVI-wiki[5]).

The Colocalization Analyzer is an extended optional tool, and is enabled by a `C` flag in the license string (see *License string details* on page 41).

Iso-colocalization object analysis

One of the features of the colocalization analyzer is iso-colocalization object analysis. It allows you to quickly determine the properties of the different colocalization regions in your data. This is realized by visualizing the colocalization map as iso-colocalization surfaces. In this way regions in which the degree of colocalization exceeds a certain value become objects. By clicking on the objects local colocalization parameters are computed and reported. To relate the iso-colocalization objects to the original data the surface objects can be blended with a MIP projection of the data (p. 20). The color range in which these objects are displayed can be modified using a hue selector (p. 24).

How to use the Colocalization Analyzer

To start the Colocalization Analyzer in Huygens Essential, right-click on an image's thumbnail to open the contextual menu, then select SHOW IN COLOCALIZATION ANALYZER. Alternatively, you can select an image's thumbnail, then in the menu bar select VISUALIZATION > LAUNCH COLOCALIZATION ANALYZER. The image must be multi channel (see p. 17) as the colocalization is based on the overlapping of different channel intensities. Wait for the analyzer to initialize and to compute the first MIP projection of the data.

You can select the viewpoint of the MIP projection by moving the TILT and TWIST slides, or by dragging the mouse pointer on the object view. Also try changing the zoom.

5 http://support.svi.nl/wiki/ColocalizationTheory

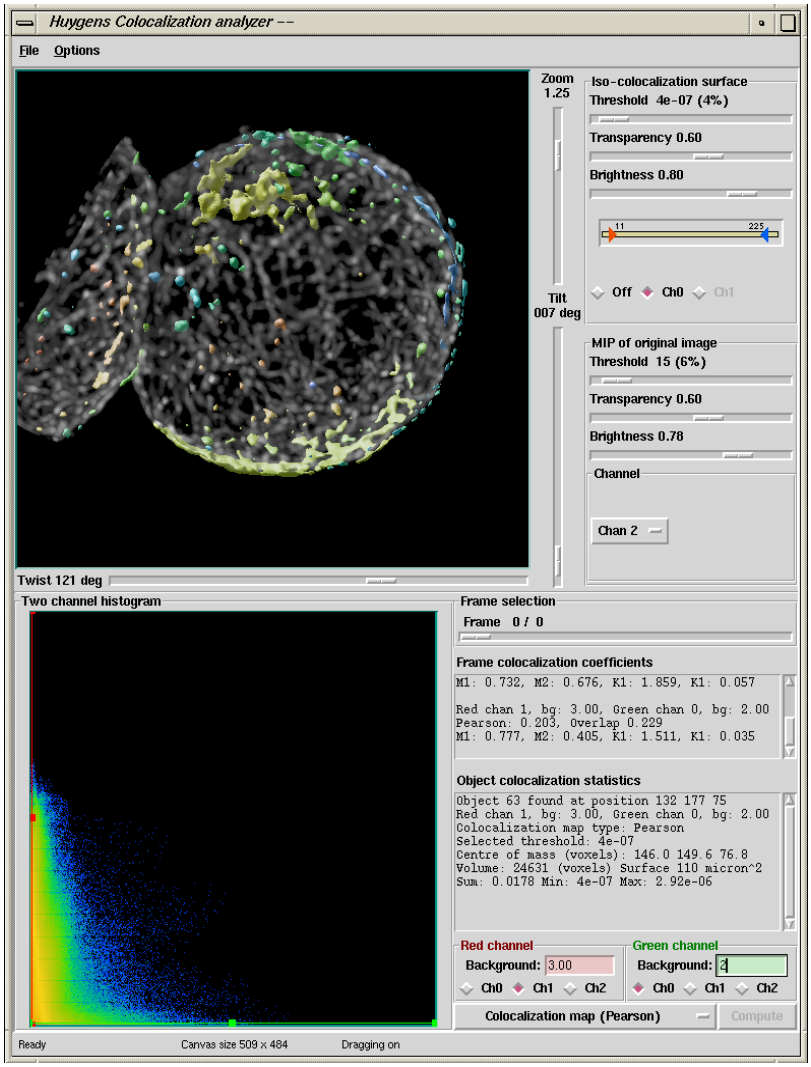


Figure 27. ***Colocalization Analyzer*** *with surfaces for regions of 'overlapping'.*

At the beginning the iso-colocalization surface sliders are deactivated, as we have to calculate a colocalization map first.

First we select the data to analyze. For time series, the "frame selector" selects the time coordinate.

We follow the usual naming convention in colocalization theory for the two compared channels: Red for the first channel, Green for the second channel. We can select, in the lower part of the window, which data channels from our image are the Red and Green channels to be compared.

A two-channel histogram is calculated by default, and updated whenever we change the Red or Green channel selection. This histogram is already an indication of the degree of overlapping between the selected channels: for two channels with a high degree of overlapping, the histogram pixels trend to concentrate along the diagonal $y = x$ line. On the other side, total absence of overlapping would produce a 2D histogram with values only on the coordinate axes[6].

By moving the colored threshold lines in the histogram, or by changing the numeric values in the input fields, we specify the backgrounds for the two selected channels. These values are subtracted from the voxels intensities when calculating the coefficients (if the result is negative, it is understood as a zero). Generally the colocalization coefficients depend much on correct estimation of the image background and resolution. For these reasons we strongly recommend to compute colocalization coefficients only on deconvolved images.

6 Read more on `http://support.svi.nl/wiki/TwoChannelHistogram`

Next we select what colocalization map we want to calculate: Pearson, Overlap, Manders "M", "K", or "H". In the computation of Manders coefficients the background values act like a threshold: to the computation of m'_1_' only pixels in R contribute when their corresponding pixel in G is above the threshold.

Finally we click on COMPUTE.

The *Frame colocalization coefficients* pane will show all available colocalization coefficients for the whole frame.

The obtained colocalization map is represented in the renderer window by iso-colocalization surfaces: regions in which the degree of colocalization exceeds a certain value become objects. This "certain value" can be controlled by the threshold slider in the iso-colocalization surface parameters. You can control the transparency and the brightness of this surface pipe with the correspondent sliders, or just set this pipe off. The color range in which the objects are displayed can be modified using a hue selector (see page 24).

Some modes generate two-channel colocalization maps: colocalization of Red respect to Green, and vice versa, e.g. in case of the Manders M1 and M2 coefficients. In these cases, the iso-colocalization surface parameters will offer the possibility of rendering any of the two channels, and thus the threshold is referred to the active one.

By clicking on the rendered objects local colocalization parameters are computed and reported.

There is one maximum intensity projection (MIP) pipe available to redirect one of your data channels to. The MIP rendering of one original channel can be a good spatial reference for the objects from the colocalization map. Use the threshold slider to select what voxels are considered for the MIP rendering, depending on their intensities. As with the surface pipe you can also control the transparency and the brightness of this MIP.

All the obtained information can be saved to external data (text or image) files through the FILE menu.

6. The PSF Distiller

Starting from version 2.5.7 Huygens Essential is optionally equipped with the *PSF Distiller* to allow you to measure your microscope's PSF. Measured PSFs allow you to improve deconvolution results and may also serve as a quality test for your microscope. It is a calibration of the microscope, in the sense of relating a physical known object with what the microscope actually measures.

The PSF Distiller is an optional component enabled by a `f` flag in the license string. Please refer to the *License string details* on page 41.

The PSF Distiller is an intelligent wizard based tool that is able to measure a PSF from one or more images of fluorescent beads, each containing one or more beads. It is also able to distill multi-channel PSFs from information collected from multi wavelength beads, or assemble a multi-channel PSF from single channel PSFs.

The PSF Distiller wizard leads you through four stages to carry out the following tasks:

- Preprocessing 'P'-stage to check microscopic image parameters. This stage is similar to the deconvolution 'P' stage.
- Stage 1: Averaging stage. In this stage all channels of the image are searched for beads that meet the selection criteria. After each successful or unsuccessful search there is the possibility to load in additional bead images, or go to the next stage.
- Stage 2: Distill stage. In this stage the PSF is measured from the averaged beads, for all available channels.

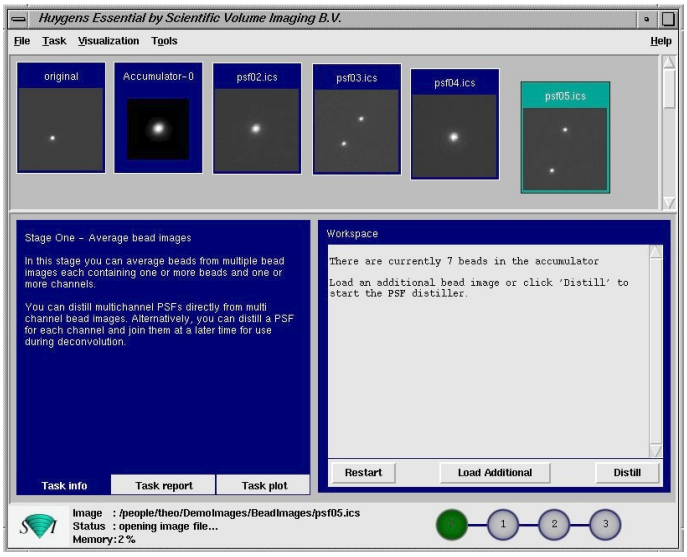

Huygens Essential by Scientific Volume Imaging B.V.
File Task Visualization Tools Help
original
Accumulator-0
psf02.ics
psf03.ics
psf04.ics
psf05.ics
Stage One - Average bead images
In this stage you can average beads from multiple bead images each containing one or more beads and one or more channels.
You can distill multichannel PSFs directly from multi channel bead images. Alternatively, you can distill a PSF for each channel and join them at a later time for use during deconvolution.
Workspace
There are currently 7 beads in the accumulator
Load an additional bead image or click 'Distill' to start the PSF distiller.
Task info
Task report
Task plot
Restart
Load Additional
Distill
Image : /people/theo/DemoImages/BeadImages/psf05.ics
Status : opening image file...
Memory: 2 %
1
2
3

Figure 28. ***The PSF Distiller.*** *After adding one or more bead images, an 'average bead' image is displayed in the Accumulator thumbnail. After pressing the* DISTILL *button, a PSF will be generated in a PSF thumbnail.*

- Stage 3: Assembly stage. In case you want to combine results from earlier Distill runs with the current result to obtain a multi channel PSF you can add the earlier result here. It is also possible to add single or multi channel earlier results to a current multi channel result.

In the section *The Distiller stages* below, the stages will be discussed in detail.

Beads for PSF distillation

The diameter of the beads should be in the order of the Half Intensity Width of the expected PSF. Larger beads will reduce the accuracy of the Distiller; smaller beads yield insufficient signal for accurate stacking in the averaging procedure, also resulting also in reduced accuracy. Typically beads with a diameter of 160 nm perform very well for many types of microscopy.

Beads should be recorded with **the same** microscopic parameters that you will use later to image your specimens. Please find more information about beads for PSF measurements on the SVI-wiki, at `http://support.svi.nl/wiki/RecordingBeads`.

The Distiller stages

Starting the Distiller

After launching Huygens Essential open the first bead image with FILE > OPEN. If your license includes the PSF Distiller option the START PSF DISTILLER button appears in the workspace; press it. One or more *Accumulator* images will be created into which later on the averaged beads will be kept. You now enter the 'P' stage.

Parameter check stage

This stage is similar to the deconvolution 'P' stage and allows you to check all relevant microscopic parameters, in particular the sampling density.

Do not use undersampled bead images!
If any of the entry fields for the sampling density turn orange or worse, red, the data in unusable!

The parameters of bead images loaded at a later stage should match the ones you establish in this stage; if they do not you will be warned.

Averaging stage

After setting the diameter and estimated Signal to Noise Ratio (SNR) of the beads, the image is searched for beads which meet all selection criteria:

- A bead should not be too close to another bead. If a bead it too close to another bead their images will interfere with each other. In widefield bead images this is quite problematic due to the large size of the blur cone. Fortunately, widefield PSF can be derived from single bead images.
- A bead should not be too close to an image edge. After all, another bead might be located just over the image edge.
- The intensity of a bead should not deviate too much from the median intensity of all beads. If it is brighter then it may be an cluster of two or more beads. If it is dimmer then it is not likely to be a bead. In both cases the object geometry is unknown so they are unusable.

If for some reason no beads are found, an explanation and some advice will be displayed in a popup. The software will try to find beads first with ideal selection criteria. If this does not yield a single bead then it will automatically retry with reduced inter bead distance criteria.

Confocal and two photon bead images

Images from 160 nm beads should look like smooth fuzzy blobs with hardly visible noise. Use the default SNR settings. If available, it is a good idea to average 2−5 beads. Two photon bead images may look slightly noisy. If so, set the SNR to 20 and average 4−10 beads. To load additional bead images press LOAD ADDITIONAL after completing a search.

Widefield bead images

Images from 160 nm beads should look like smooth fuzzy blobs with no visible noise. Use the default SNR settings. It is not necessary to average any more beads.

After you have added the last bead recording press DISTILL to enter the Distillation stage.

Distillation stage

The distillation stage usually requires no user intervention, though in some cases a popup will be displayed with a question or a warning. All channels will be processed automatically.

Assembly stage

In the assembly stage you can add a previously obtained PSF image file as a channel before or after the current result. Press ADD CHANNEL if you want to do this. The Distiller will compare the microscopic image parameters of the selected PSF image and check its content. In case there are

problems the software will ask you to decide between ignoring the differences and discarding the selected file.

If you do not want to add channels press NEXT and then DONE. This leaves you with a loaded measured PSF for immediate use in a subsequent deconvolution run.

We advise you to measure the PSF of your system for each recording situation and certainly after each maintenance job in which the optics or scanning device was serviced.

Read more on the SVI-wiki, at http://support.svi.nl/wiki/PsfDistiller.

7. Establishing image parameters

The deconvolution algorithm needs to know some of the parameters describing your image acquisition. These are not too many, but you should carefully determine them. They are explained in this chapter.

Image size

The amount of computing time involved in deconvolving images is more than proportional to the image size. It is therefore sensible to limit the data size as much as possible. With widefield images we recommend to not record planes below and above the object which only contain blur. Huygens Essential does not need these planes to restore your object. Since the blur in these planes might be affected by hard to correct bleaching they might even reduce the quality of the deconvolution result. In any case, never crop your objects of interest. As a rule of thumb, leave about one extra micron above and below your objects.

Brick wise processing

Deconvolving images requires much computer memory because all computations are done in 32-bit floating point format, and because several extra (hidden) images are needed to store intermediate results. To reduce the memory requirements Huygens Essential will split your images into bricks, deconvolve the bricks sequentially, and fit the bricks together in a seamless fashion. Brick wise processing is an automatic feature of Huygens Essential. To find out the best number of bricks, let the software run in automatic mode for splitting. It will consider many options and go for the best one.

Signal to Noise Ratio (SNR)

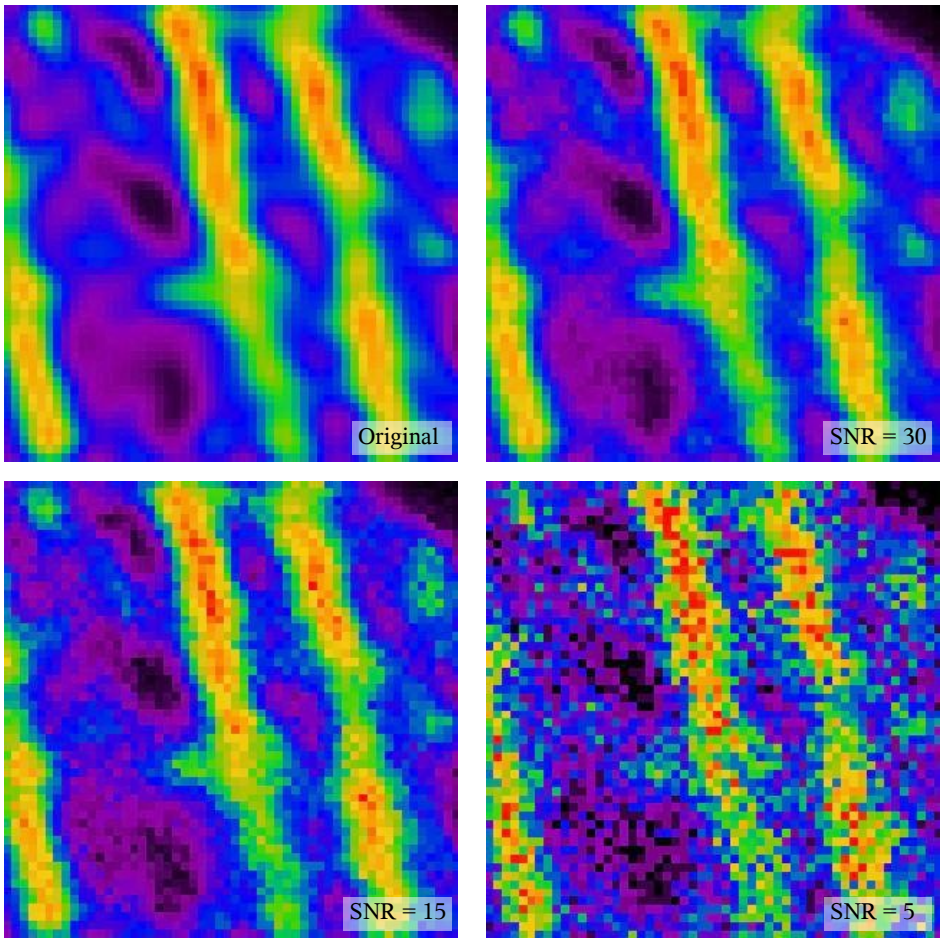


Figure 29. ***Examples of different SNR values.*** *Same image, different SNR values. Top left: original image. Top right: image with SNR = 30. Bottom left: image with SNR = 15. Bottom right: image with SNR = 5.*

The Signal to Noise Ratio (SNR) can be estimated from the quality of the image. In Figure 29 you find some examples of recordings where different noise levels were added to an original (restored) image.

If you want to calculate this SNR with more detail, you must remember that we **define the SNR** as the square root of the number of photons in the brightest part of the image.

Estimating the SNR in noisy images is surprisingly easy. It is based on the idea of establishing the voxel intensity value *s* corresponding to a single photon 'hit', by looking for such an event in dark areas of the image. Knowing the intensity value *M* of the brightest voxel in the image, one can now calculate how many photons are involved in it. Thus the SNR is

$$SNR = \sqrt{\frac{M}{s}}$$

If a significant blacklevel is present, it should be subtracted from these values before making the quotient. See the paragraph *Blacklevel* below for more details.

In low noise images it is much more difficult, as you no longer see single photon events so easily. Fortunately, in such cases the establishment of the precise SNR is not very important for the res-

toration method, and a rough estimation based on the appearance of the image is usually enough (see Figure 29).

Read more in http://support.svi.nl/wiki/SignalToNoiseRatio.

Blacklevel

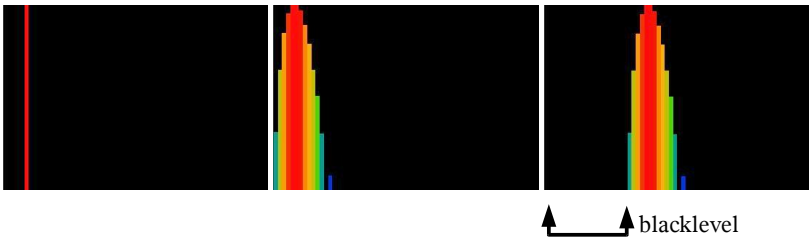


Figure 30. ***Histogram of images with various blacklevel values.***

Figure 30 shows the histograms of three synthetic images. At left an image homogeneously filled with the value 5. At the middle we applied Poisson noise as if the image was build by a CCD camera. At the image on the right a value of 20 was added to simulate electronic shift. This shift is called 'blacklevel'. A large blacklevel value will reduce the effective dynamic range of your microscope, but will do no harm to the deconvolution since it is automatically accounted for in the background estimation stage. However, it is also possible that the blacklevel is negative. In the image histogram this will show up as a spike on the left.

Sampling densities

It is very important for the quality of a deconvolution result that all information generated by the optics of the microscope is captured in digital form. It can be shown that if the sampling density is higher than a certain value all information about the object is captured. We will call this value the critical sampling distance, corresponding to the Nyquist rate. Apart from practical problems like bleaching, acquisition time and data size there is no objection at all against using a smaller sampling distance than the critical distance, to the contrary.

Figure 31 shows the dependency of this critical sampling distance on the *NA* for specific wavelengths. To apply the plot of Figure 31 to different wavelengths you can simply scale the vertical axis with the wavelength. Example: you are working with a WF microscope with *NA* 1.3 at emission wavelength 570 nm. From the plot you read that the critical lateral Nyquist sampling distance at 500 nm emission is 95 nm, so in your case this becomes 570 / 500 × 95 nm = 108 nm.

In the confocal case it is the excitation wavelength which determines the Nyquist sample distance. In theory the pinhole plays no role, but larger pinholes strongly attenuate fine structures at the resolution limit. Therefore, as a rule of thumb, with a common pinhole diameter of 1 Airy disk the lateral critical sampling distance may be increased by 50% with negligible loss of information. In cases were the pinhole is much larger, the lateral imaging properties much resemble those of a WF system and the sampling distance can be set accordingly. We do not recommend to increase the axial sampling distance appreciably beyond the critical distance

In a multi-photon excitation microscope, it is the excitation wavelength divided by the photon count which determines the sampling.

Read more on the SVI-wiki: http://support.svi.nl/wiki/NyquistRate.

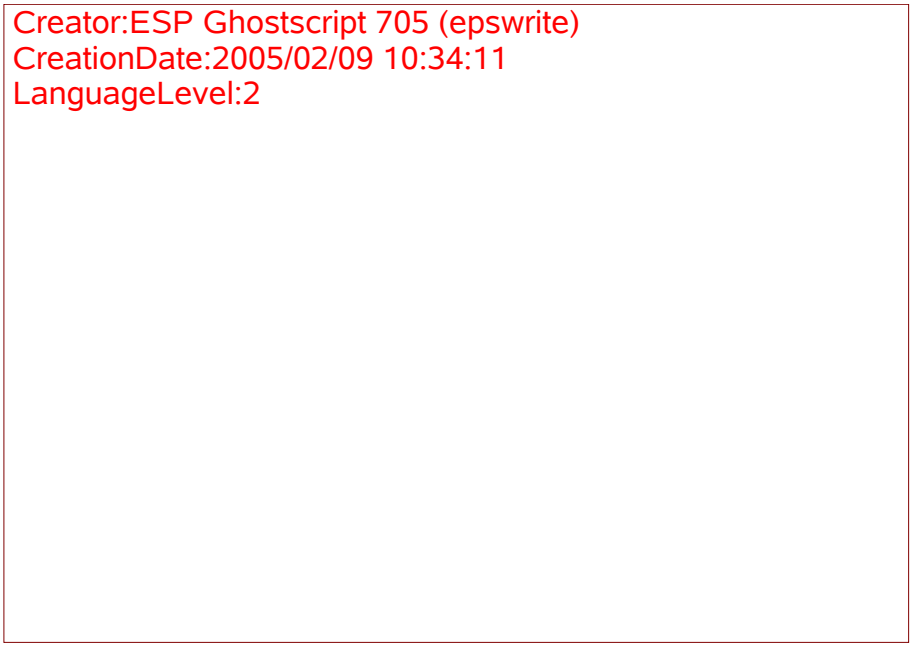


Figure 31. ***Critical sampling distance vs. NA.*** *The curves above show the critical sampling distance in axial and lateral directions for wide-field and confocal microscopes. The emission wavelength in both cases is 500 nm.*

Computing the backprojected pinhole radius

Throughout the Huygens Professional and Huygens Essential pinhole sizes of confocal systems are specified as the **backprojected radius in nanometers** r_b. 'Backprojected' means the size of the pinhole as it appears in the specimen plane: the physical pinhole radius r_{phys} divided by the total magnification of the detection system. This total magnification is the product of the (variable) objective magnification times a fixed internal magnification:

$$r_b = \frac{r_{phys}}{m_{obj}\ m_{system}} \tag{1}$$

where m_{obj} is the magnification factor of the objective and m_{system} is the fixed magnification of the system.

The equations that you can find in the next pages for different microscopes are intended to orientate the user in finding out the backprojected value, but the idea is always the same: given the *diameter* of the real pinhole *d*, we apply a factor for unit conversion (to obtain the *radius* in nm), and some dividing numbers that take account of the magnification of the microscope. These include both the objective and the intrinsic system magnification. In some microscopes with pinhole shapes other than circular, a geometrical correction will also be needed.

Airy disk as unit for the backprojected pinhole

Some confocal microscopes report the pinhole size (diameter) with the Airy disk (diameter) as unit. The backprojected pinhole radius can then be computed with:

$$r_b = \frac{0.61\ \lambda_{ex}\ N_{Airydisks}}{NA} \tag{2}$$

with *NA* the numerical aperture of the lens, $N_{Airydisks}$ the number of Airy disks, and λ_{ex} the excitation wavelength. In principle using λ_{ex} is not correct because the Airy diffraction pattern is formed by the emitted light. However, we suspect microscope manufacturers prefer to use the excitation wavelength because it is better defined and does not depend on settings of devices like adjustable band filters. For this reason in the formula above we too use λ_{ex}.

Note that this relation bypasses the need to know internal system and lens magnifications.

Converting from integer parameter

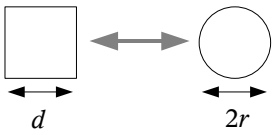
Unfortunately, quite a few commercial microscopes do not report the physical pinhole size or the Airy disk size. Instead, often an integer size parameter is specified with a range 0−255 (8-bit). Matters are further complicated by the use of non-circular pinholes. To compensate this we introduce a shape factor c_{shape} which takes care of the conversion from *size* (diameter or edge size) to *radius*. The following formula can be used to translate the 8-bit machine number into the backprojected pinhole radius:

$$r_b = 1000\ c_{shape}\ \frac{(P_8/255)\ (s_{max} - s_{min}) + s_{min}}{m_{obj}\ m_{system}} \tag{3}$$

where P_8 is the 8-bit machine number for the pinhole, s_{min} is the maximal pinhole size in *micrometers*, s_{min} is the minimal pinhole size in micrometers. The factor 1000 is to convert from microns to nanometers.

Shape factor

The shape correction from a square to a circular pinhole is based on setting the area of the square pinhole equal to the area of the replacing circular pinhole.



$$d^2 = \pi\ r^2 \text{ and thus } r = \frac{d}{\sqrt{\pi}} \tag{4}$$

For a square pinhole $c_{shape} = 1/\sqrt{\pi} = 0.564$. For circular pinholes $c_{shape} = 0.5$ to just convert from diameter to radius.

Airy disk as unit for the backprojected radius of a square pinhole

The relation between the edge size of square pinhole in Airy disk units and the backprojected radius is a combination of equations 2 and 4. Taking into account that equation 2 already converts a diameter into a radius, we get:

$$r_b = \frac{0.69\,\lambda_{ex}\,N_{Airydisks}}{NA} \tag{5}$$

with $N_{Airydisks}$ the number of Airy disks.

Computing the backprojected pinhole distance in Nipkow spinning disks

As is the case for the backprojected pinhole diameter, the distances between the pinholes in spinning disks must be divided by the system magnification. For the most used Yokogawa, for example, the pinhole distance is 2.5 µm. You can check this by stopping the disk. So with an 100× lens the backprojected distance is about 2.5 µm.

Pinhole radius tables

In the case of the Leica TCS, the Biorad MRC, the Olympus Fluoview and the Zeiss LSM microscopes the pinhole geometry and system magnification is known resulting in the conversion formulas from the following tables:

Leica confocal microscopes

TCS 4d, SP1, NT

The size of the square pinhole is given as an 8-bit number which maps to the physical pinhole radius given in the following table:

TCS4d	Range begin	Range end
Reported parameter (P_8)	0	255
Diameter (micron)	20	630 (earlier reported as 500)
Pinhole geometry	square	
System magnification	4.5	
Backprojected pinhole radius (nm)	$\frac{295\,P_8 + 2508}{m_{obj}}$ (Eq. 6)	

Table 1. Leica TCS4d pinhole parameters. This table is believed to be valid for the SP1 and NT models too.

If the pinhole is specified in Airy disk units, see *Airy disk as unit for the backprojected radius of a square pinhole* on page 34 for information on how to convert to a backprojected radius.

TCS-SP2

The Leica TCS-SP2 has a system magnification of 3.6. However, the size of its square pinhole is usually specified in Airy disk units making it independent of the actual overall magnification. It is dependent though on wavelength and *NA* of the objective. See *Airy disk as unit for the backprojected radius of a square pinhole* on page 34 for information on how to convert to a backprojected radius.

Zeiss confocal microscopes

Zeiss LSM410 inverted	Range begin	Range end
Reported parameter (P_8)	0	255
Diameter (micron)	0	1000
Pinhole geometry	square	
System magnification (m_{sys})	2.23	
Backprojected pinhole radius (nm)	$\frac{992\ P_8}{m_{obj}}$ (Eq. 7)	

Table 2. Zeiss LSM410 (inverted) pinhole parameters.

Zeiss LSM510	
Diameter (micron)	d
Pinhole geometry	circular
System magnification (m_{sys})	3.33
Backprojected pinhole radius (nm)	$\frac{10^3\ d}{2\ m_{sys}\ m_{obj}}$ (Eq. 8)

Table 3. Zeiss LSM510 pinhole parameters.

Olympus confocal microscopes

Olympus Fluoview					
Reported parameter	1	2	3	4	5
Diameter d (micron)	0	100	150	200	300
Pinhole geometry	square				
System magnification (m_{sys})	3.426 / 3.8 (FV500)				
Backprojected pinhole radius (nm)	$\frac{10^3\ d}{2\sqrt{\pi}\ m_{sys}\ m_{obj}}$ (Eq. 9)				

Table 4. Olympus Fluoview pinhole parameters.

Biorad confocal microscopes

Biorad	
Reported parameter (P_8)	n.a.
Diameter d (mm)	0–8
Pinhole geometry	circular
System magnification (m_{sys})	53–83, reported 60 for the Radiance, 53 for the 1024.
Backprojected pinhole radius (nm)	$\frac{10^6\ d}{2\ m_{system}\ m_{obj}}$ (Eq. 10)

Table 5. Biorad MRC600/1024/Radiance pinhole parameters.

Checking the Biorad system magnification

The Biorad MRC500/600/1024 microscopes have a very high magnification in the detection system. The fixed system magnification is, according to Pawley[7], 53 × m_{tube}, with m_{tube} between 1.0 and 1.56 (a factor 1.25 for the fluorescence attachment and also a factor 1.25 for the 'DIC' attachment). The factor of 53 includes the 8× "eyepiece" just below the scan head, but does not include that variability in magnification due to the variations in tube-length that are result from the aligning the system.

The high system magnification allows you to view the diffraction pattern (Airy disk) at the pinhole plane directly by eye. To enable you to verify the correctness of equation 10 in the table above for your instrument we outline the way the system magnification was derived:

In a Biorad MRC600 with a 1.3 60× objective, the Airy disk has a diameter of around 2−2.5 mm at the pinhole plane. The diameter of the first Airy zero ring is 7.6 lateral optical units (o.u.), using the following transformation to express a distance x in dimensionless o.u.,

$$v = x \frac{2\pi}{\lambda} n \sin\alpha \tag{11}$$

where $n\sin\alpha$ is the numerical aperture *NA*. In the system above an o.u. is 0.3 ± 0.033 mm. At the specimen plane (backprojected) a lateral o.u. is in this case around 61 nm. The total magnification appears thus to be 4918, the system magnification 82 ± 9. This value corresponds well with the largest possible system magnification for the MRC600.

A supplied calibration curve

If a calibration curve was supplied with your microscope best use that curve to convert the displayed setting to a physical size and from there convert to the backprojected radius.

7 Pawley, J. B. Handbook of biological confocal microscopy, 2nd ed. 1995. Plenum Press, New York and London. ISBN 0-306-448262. Page 30.

8. Improving the quality of your images

In this chapter you can find some basic suggestions on how to acquire better microscope images. They are based on frequent problems that we find on users data. These recommendations go on the direction of obtaining the greatest quality images from your microscope, from the point of view of acquiring as much information as clean as possible. This alone worths the effort, but it will also be very valuable for the deconvolution afterwards.

Some basic guidelines to improve the deconvolution results are also included. You can find more information at the SVI-wiki, at `http://support.svi.nl/wiki`.

Data acquisition pitfalls

Some of the most common problems arising when acquiring images are addressed in this section.

Refractive index mismatch

A mismatch between the refractive index of the lens immersion medium and specimen embedding medium will cause several serious problems:

- Geometrical distortion: the fishtank effect
  Objects will appear elongated in the microscope. Huygens Essential takes this into account when calculating the theoretical PSF, but it will not modify the image geometry.
- Spherical aberration (S.A.)
  S.A. will cause the oblique rays to be focused in a different location than the central rays. The distance in this focal shift is dependent on the depth of the focus in the specimen. If the mismatch is large, e.g. when going from oil immersion into a watery medium, the PSF will become asymmetric at depths of already a few micron. Especially harmful for WF deconvolution. Workaround: keep the *z*-range of the data as small as possible. Solution: use a water immersion lens.
- Total internal reflection
  When the lens numerical aperture *NA* is larger than the medium refractive index *n* total internal reflection will occur, causing excitation light to be bounced back into the lens and limiting the effective *NA*.

If S.A. is unavoidable, you can still improve the image during restoration using an adaptive point spread function. See *Spherical aberration correction* on page 40.

Clipping

The light intensities from the microscopic object are converted to electrical signals that pass an adjustable amplifier. Also an electrical DC component can be added or subtracted by the microscope operator. The electrical signal may thus range from negative to highly positive. These electrical signals must be converted to numbers processed by the computer. This converting stage is done in the CCD camera and its electronics. Most CCD cameras have an 12-bit converter limiting the output numbers to a range of 0 to 4095. Negative input signals are usually converted to 0 while positive input values exceeding some value are all converted to 4095 (clipping): information in the clipped samples is *lost*.

In practice: be suspicious if you find in your image voxel intensity values at the extremes of the numerical range, probably clipping occurred.

Undersampling

One of the rules of measurement that is often overlooked is that one takes too few *xy* slices from the microscopic object. In that case the sampling distance is too large (too few samples: under-sampled) which leaves you with a 3-D stack with hardly any relation between the adjacent planes. It is important to know how the sampling conditions should be established in order to recover an image from the sampled values. How you should sample your object depends on your microscope type -WF or confocal- and on the microscope parameters used, like the numerical aperture and wavelength of the light. The correct sampling distances can be estimated as explained on *Sampling densities* (page 32) or calculated using the formulas as given in *The Nyquist rate* from the **Huygens Deconvolution Recipes**[8].

Do not undersample to limit photodamage

Some times undersampling is done to limit photodamage to live cells. If photodamage plays a role it is better to distribute the available photons over more pixels, resulting in an apparently noisier image, than putting the photons in fewer pixels to get a low noise, but undersampled, image. Of course there are limits, but a fair trade-off can be often found.

It is better to record 10 separate noisy slices 100 nm apart than slices on 1000 nm each averaged 5 times in order to reduce noise. See also *A typical example* in *Sampling an image* from the **Huygens Deconvolution Recipes** or in the above mentioned Internet link.

Bleaching

Bleaching is a practically unavoidable phenomenon in fluorescence microscopy. Because the image planes are acquired sequentially, bleaching will vary along the *z* direction. Assuming it is not strong it will not affect deconvolution results on confocal or two photon images. But in WF deconvolution bleaching is more of a problem. Fortunately, usually the bleaching in WF images can be corrected quite easily. Huygens will do so automatically. However, if the bleaching is strong the correction might not be perfect, resulting in lower quality deconvolution results.

Illumination instability

Some WF systems are equipped with unstable arc lamps. Huygens will correct this instability, but when the instability is severe it cannot do so sufficiently.

Mechanical instability

Mechanical instability can take many shapes, for example:

- *Vibrations sometimes seen in confocal images*. They may seriously hamper deconvolution
- *z-stage moves irregular or with sudden jumps*. Fatal for confocal or WF deconvolution.
- *Specimen moves.* If in WF data the object can clearly be seen moving when slicing along over a few micron in *z* this will cause problems for the deconvolution. Best cause of action, apart from speeding up acquisition, is limiting the *z*-range of the data as much as possible. Confocal data of moving specimen causes less problems.

Thermal effects

Thermal effects are known to affect calibration of the *z*-stage, especially if piezo actuators without feedback control are used. In particular harmful for WF data.

8 Also available on-line in `http://support.svi.nl/wiki/NyquistRate`

Internal reflection

At high *NA* the angle of incidence of the most oblique rays can be close to 70 degrees. When a ray has to cross the cover-glass to medium interface at such an angle total reflection may occur. To be precise, total reflection occurs when the *NA* of your lens is higher than the refractive index of the embedding medium. This will reduce the effective *NA* of the lens.

Deconvolution improvements

Acquire an experimental PSF

A point spread function (PSF) is the image of a single point object. The degree of spreading (blurring) in the image of this point object is a measure for the quality of an optical system, and the imaging in a fluorescent microscope is completely described by its PSF. Although in many cases a theoretically calculated PSF very well matches the real one, ideal theoretical calculations can not predict actual misalignments or other problems inside the optical path. Therefore it is always recommendable to measure an experimental PSF and, if it is very different from the ideal one, use it for deconvolution instead of the theoretical one.

Obtaining an experimental PSF is based on recording the image of a well know probe, as close as the ideal subresolution light source as possible, and distilling a PSF from it using *The PSF Distiller* as explained on page 27.

Spherical aberration correction

The Huygens software automatically adapts the **theoretical** point spread function (PSF) to the sample depth to correct for spherical aberration, in case of a refractive index mismatch. For that, the program considers that the coverslip is placed at the bottom of the image, at lower Z coordinates (as in an inverted microscope). If your image suffers from a refractive index mismatch you should adapt the image to this condition before the restoration: the Huygens Essential has a MIRROR ALONG Z tool, to flip the image in case your coverslip is on the top.

In case your image suffers from high spherical aberration, it might be better to use a theoretical PSF with this depth-dependent correction than an experimental one.

Improve the estimated parameters

This is an usual procedure to improve the estimated parameters for deconvolution: you start out with a signal-to-noise ratio (SNR) derived either from an estimate of the number of photons in the image or simply from a visual inspection (as explained on page 31). Subsequently you do a restoration run and inspect the result for artifacts and residue background. If you are confident all is fine you resume the restoration with a higher SNR setting (say 30%-50%) and perhaps a higher background. The software will ask you to continue were you left off (keeping improving the image, quite recommendable) or to start from the raw image again with the new parameters. A new result will be generated to compare with the previous one, for instance using the Twin Slicer. You can repeat this several times, and at the end you will be asked to select the best result as the final one.

If you have done this a previous time for a similar image then of course you just use the values established then!

9. Appendix

License string details

You can have detailed information about your license strings by going to HELP > LICENSE, select the license string you want to inspect, and click EXPLAIN LICENSE.

This is a License String example:

```
HuEss-2.7-wcnp4-d-ft-demnps-2008Sep30-33dfa680a8402167-
{info@svi.nl}-c338a4da57cb342151d3
```

As from Huygens Compute engine version 2.4 the license strings have the following format:

```
<prod>-<vers>-<micr>-<server>-<flags>-<lock>-<date>-<sysid>-
<email>-<checksum>
```

Where:

`<prod>` can be one of HuEss, HuScript, HuPro, FluVR.
`<vers>` is the version number which is 2.4 or higher.
`<micr>` consists of one or more characters representing microscope types:

`w`: Widefield, enables you to deconvolve WF data
`c`: Confocal, enables you to deconvolve confocal data
`n`: Nipkow Disk, enables you to deconvolve data from Nipkow (spinning) Disks
`p`: multi-photon, for 2-(or more) photon microscopes
`4`: 4-pi, for experimental microscopes

`<server>` can be one of:

`d`: desktop (1 or 2 CPU's)
`s`: small server (3−8 CPU's)
`m`: medium server (9−32 CPU's)
`l`: large server (33−64 CPU's)
`x`: extreme server (65 or more CPU's)

Remark for Linux and Mac OS X multi-processing users:

`d`: desktop (1 thread)
`m`: medium server (max 2 threads)
`l`: large server (max 4 threads)
`x`: extreme server (5 threads and more)

`<extra flags>` which enables additional functionality:

`f`: Distiller option, enables obtaining PSF's from measured beads.
`t`: Time series option, enables deconvolution of time series, either 2D-time or 3D-time
`v`: Surface Renderer visualization option.
`C`: Colocalization Analyzer option.
`A`: Object Analyzer option.
`H`: 64 bit.

<lock> Set of license policy or locking flags having of one or more characters:

d: License expiry date
e: Email address
m: Hardware system ID
n: Number of processors
p: Processor type
s: Processor details

<date> is the end of license or end of support date in the format eomYYYYMMMDD
<sysid> is 16 hexadecimal digits of system identification
<email> is the email address of the customer who bought this license in the format {email address}
<checksum> is 20 hexadecimal digits of license checksum

When the d flag (license expiry date) is specified, then after the locking flags comes the expiry date. Without this flag the expiry date will be preceded with the letters eom, which turns the date into an end date of the current support contract. Currently the e flag is always present. If it is after the system ID an email address surrounded by braces is given. This is the email address of either the creator of the license, or the sales person in case of a temporary license, or the customer to whom the license was sold. This is used for informational purposes only. It does not limit the license in any way. If the m flag is present then the hardware system identification ID must match the one from the running system. Similar for the n flag. It requires that the number of processors as specified by the system ID matches the number in the current system. The p requires a match on processor type. For PCs this consists of the name of the processor vendor like Intel or AMD, the processor family like Pentium, and the processor model. For Irix machines this is the IP level of the processor board which can be found in the first line of the output of the hinv command. The s flag is only meaningful for PCs. It enables additional matching on the processor core 'stepping number' which is a notation from Intel (like a revision number) that indicates the changes or improvements inside the CPU's instructions set.

Questions

Where can I find support on the web?

See *Addresses and URLs* on page 45.

What does the quality factor mean while running Huygens?

Deconvolution as it is done in Huygens Essential hinges around the idea of finding an as good as possible estimate of the object that is imaged by the microscope. To assess the quality of an estimate, Huygens Essential computes the image of each estimate as it would appear in the microscope and compares it with the measured image. From the difference a quality factor is computed. The difference is also used to compute a correction factor to modify the estimate in such a way that the corrected estimate will yield a better quality factor. The quality factor as reported by Huygens Essential is a measure relative to the first estimate and therefore a number greater or equal to 1. If the increase in quality drops below a threshold the iterations are stopped. See also *Quality threshold* on page 15.

Can I deconvolve a TIFF series?

Yes, if the series is a numbered series like: slice001.tif, slice002.tif, ..., slice0nn.tif Huygens Essential will read the series into a single 3D image. Because TIFF's usually carry no additional microscopic information, check the parameters carefully.

TIFF file series naming convention

If you have TIFF images to be read into the Huygens Professional or the Huygens Essential you should know about the naming convention used.

If you select a file from a numbered series, the selected file and the following files will be interpreted as x-y planes of a 3D stack and read into a 3D image of suitable size and channel configuration.

A one-channel 3D images only go with numbers:
As an example a dataset called `c` with 32 slices numbers as follows:

```
c000.tif
c001.tif
...
...
c031.tif
```

If you wish to work on the complete `c` stack you only have to select `c000.tif` while opening. If you select file `c020-tif` the first 20 slices will be skipped.

Numbered series without the TIFF extension like

```
c04
c05
...
...
c18
```

are not read in as a series.

Huygens Essential and Professional read and write TIFF series with Leica style numbering if there are more channels (different wavelengths), slices or frames(in time).

An image of four slices and two frames is named with Leica style numbering as follows:

```
c_t00_z000.tif
c_t00_z001.tif
c_t00_z002.tif
c_t00_z003.tif
c_t01_z000.tif
c_t01_z001.tif
c_t01_z002.tif
c_t01_z003.tif
```

And an image `sTCh` of four slices, three frames and two channels:

```
sTCh_t00_z000_ch00.tif
sTCh_t00_z000_ch01.tif
sTCh_t00_z001_ch00.tif
sTCh_t00_z001_ch01.tif
sTCh_t00_z002_ch00.tif
sTCh_t00_z002_ch01.tif
sTCh_t00_z003_ch00.tif
sTCh_t00_z003_ch01.tif
sTCh_t01_z000_ch00.tif
sTCh_t01_z000_ch01.tif
sTCh_t01_z001_ch00.tif
sTCh_t01_z001_ch01.tif
```

```
sTCh_t01_z002_ch00.tif
sTCh_t01_z002_ch01.tif
sTCh_t01_z003_ch00.tif
sTCh_t01_z003_ch01.tif
sTCh_t02_z000_ch00.tif
sTCh_t02_z000_ch01.tif
sTCh_t02_z001_ch00.tif
sTCh_t02_z001_ch01.tif
sTCh_t02_z002_ch00.tif
sTCh_t02_z002_ch01.tif
sTCh_t02_z003_ch00.tif
sTCh_t02_z003_ch01.tif
```

Can I deconvolve a single plane widefield image?

Yes. Single plane WF deconvolution works because the data is extrapolated into a region above and below the plane spanning typically between 10−20 planes of 100−300 nm sampling in *z*. The software generates an appropriate PSF.

Can I deconvolve a single TIFF image?

Yes. Huygens Essential treats the image as the only known plane of a 3D stack and proceeds as usual. Set the *z*-sampling distance to the Nyquist value: see *Sampling densities* on page 32.

How do I generate a debug log?

See `http://support.svi.nl/wiki/DebugMode`.

Addresses and URLs

Where can we be reached?

Scientific Volume Imaging b.v.

Alexanderlaan 14
1213XS Hilversum
The Netherlands

You can call us directly by phone:

+31 35 6859405 or ++653 345445,
or fax us at: +31 35 6837971,
or email us at: `info@svi.nl`

`http://www.svi.nl`

Distributors

A list of distributors can be found on our web site:

`http://www.svi.nl/distributors`

Support and FAQ

Knowledge database

It is good to know that an extensive support base is available where you may find answers to questions that come to front while reading this document. FAQ's are available for many items such as deconvolution and general microscopy, installation, memory management, visualization, file formats, platforms and reported bugs:

`http://support.svi.nl`

On this web site you will also find a form to submit questions to SVI's support team.

The SVI-wiki

The SVI-wiki is a rapidly expanding public knowledge resource on 3D microscopy and deconvolution. Based on the WikiWikiWeb principle, it is open to contributions from every visitor. In addition it serves as a support medium for SVI customers and relations to discuss different aspects of SVI's Huygens Software. The SVI-wiki can be found in

`http://support.svi.nl/wiki`

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